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Carbon and nitrogen limitation increase chitosan antifungal activity in *Neurospora crassa* and fungal human pathogens.

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Running Tittle: Carbon and nitrogen limitation enhances chitosan antifungal activity

ABSTRACT

Chitosan is a polymer obtained by partial chitin N-deacetylation with antifungal action. This compound causes plasma membrane permeabilization in sensitive filamentous fungi and yeast. Previous studies have established that membrane fluidity together with cell energy are key factors determining chitosan sensitivity in fungi. A five-fold joint reduction of both glucose (main carbon (C) source) and nitrogen (N) increased 2-fold *Neurospora crassa* sensitivity to chitosan. We were able to link this chitosan sensitivity increase due to nutrient (C and N) limitation to an intracellular production of reactive oxygen species (ROS) and to plasma membrane permeabilization. Very low chitosan concentrations (2.5 µg ml^{-1}), in low C (0.8 g l^{-1}) and low N (0.03 g l^{-1}) containing growth medium, caused a partial membrane permeabilization and a 25% growth reduction of *N. crassa*. Increasing chitosan concentration led to full membrane permeabilization and subsequent cell death. Releasing *N. crassa* from C and N limitation reduced chitosan antifungal activity in spite of high ROS intracellular levels. When lactate was used instead of glucose, C and N limitation increased *N. crassa* sensitivity to chitosan to a larger extent (ca. 4-fold) than what glucose did. Limitation of C and N also increased sensitivity of filamentous fungi and yeast human pathogens to chitosan. For *Fusarium proliferatum,* lowering 100-fold C and N content in the growth medium, increased 16-fold chitosan sensitivity. Similar results were found for *Candida albicans, C. krusei* (fluconazole resistant) and *Cryptococcus* spp. Severe C and N limitation increased chitosan antifungal activity for all pathogens tested. Chitosan at 100 µg ml^{-1} was lethal for 8 out of 10 fungal human pathogens tested but was not toxic to HEK293 (human) and COS7 (monkey) cell lines. Besides, with intermediate C and N levels, chitosan (100 µg ml^{-1}) increased 90% survival of *Galleria mellonella* larvae infected with *C. albicans* at 24 h post-inoculation respect to controls with no chitosan. These results are of paramount importance for the development of chitosan as an antifungal compound for practical use.

KEY WORDS

Chitosan; ROS; membrane permeabilization; *Neurospora crassa; Candida* spp.; *Fusarium proliferatum.*

1 Introduction

Chitosan is a polymer obtained by partial chitin N-deacetylation (Kumar, 2000) with antifungal action (Allan and Hadwiger, 1979). Many filamentous fungi (including *Neurospora crassa*) and yeast are sensitive to chitosan (Palma-Guerrero et al., 2009; Jaime et al., 2012). However, chitosan antifungal activity differs within fungal groups (Palma-Guerrero et. al 2008). Filamentous fungi and yeast human infections are a highly relevant health issue in our society (Richardson and Lass-Flörl, 2008; Miceli et al., 2011). These fungal infections have become a leading cause of human mortality due to their increasing frequency in immunocompromised populations and the low number of antifungals available (Shapiro et al., 2011). Chitosan has a great potential to be developed as an antifungal agent to treat diseases caused by human pathogenic fungi (Calamari et al., 2011; Peña et al., 2013; Kulikov et al., 2014 and Younes et al., 2014). Therefore, it is of vital importance to fully understand its mode of action.

Chitosan causes plasma membrane permeabilization in *N. crassa* (Palma-Guerrero et al., 2009) and yeast (Jaime et al., 2012). Membrane fluidity is a key factor determining chitosan sensitivity in fungi (Palma-Guerrero et al., 2010). Cell energy and mitochondrial activity have also an important role in antifungal activity of chitosan (Palma-Guerrero et al., 2009; Zakrzewska et al., 2005). These processes are known to increase reactive oxygen species (ROS) as the main by-products (Kawaltowski et al., 2009). A hyperoxidant unstable state is reached when ROS generation surpasses the antioxidant capacity of the cell. This ability could be associated with the presence of nutrients (a source of reducing power), which would help *N. crassa* to return from a hyperoxidant to a normal, less-oxidative state (Aguirre et al., 2005).

In this study, we have analyzed the effect of the nutrient levels (carbon and nitrogen limitation) and the effect of the carbon source on sensitivity to chitosan of *N. crassa* and clinically important filamentous fungi and yeast human pathogens (*Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus* spp.), besides pathogenic *Candida* spp. (*C. krusei*, *C. glabrata* and *C. parapsilosis*) resistant to currently used antifungals such as fluconazole or echinocandins. With this purpose, we have used a high-throughput spectrophotometrical methodology adapted from techniques standardized in the Clinical and Laboratory Standards Institute (NCCLS) of the USA (Clinical and Laboratory Standards Institute, 2008). Besides, we have determined in this work the production of

intracellular reactive oxygen species (ROS) by *N. crassa* and its relation to cell membrane permeabilization by chitosan under various carbon and nitrogen regimes. We have also evaluated the impact in chitosan sensitivity of *N. crassa* of replacing glucose for lactate as the main carbon source, since the latter is less energetically efficient.

Nutrient status has a substantial effect on the susceptibility of fungal pathogens (e.g. *C. albicans*) to others antifungals (e.g. miconazole) (Ene et al., 2012a). Besides, *C. albicans* encounters carbon-poor conditions during host infection (Ramirez and Lorenz, 2007). As a consequence, *C. albicans* displays an elaborated response in the host which includes activation of pathways needed to enable the use of alternative carbon sources. For this reason, gluconeogenesis, the glyoxylate cycle, and β-oxidation of fatty acids, have been shown to be required for full virulence in several pathogenic organisms (Lorenz and Fink, 2001; Prigneau et al., 2003). We have therefore determined the role of carbon and nitrogen status in *C. albicans* infecting an invertebrate model *Galleria mellonella* (Fuchs et al., 2010) treated with chitosan and artificially inoculated with the pathogen. Finally, the compatibility of chitosan with mammalian (including human) cell lines was investigated. These studies are a key step to develop chitosan as an antifungal agent in the future.

2 Materials and methods

2.1 *Filamentous fungi and culture conditions.*

Fusarium proliferatum used in this study (CECT 20546) was obtained from the Spanish Type Culture Collection (CECT, Spain). *Aspergillus fumigatus* used in this study (ATCC16907) was obtained from the American Type Culture Collection (ATCC, USA). *Hamigera avellanea* (CECT 20819) was obtained from the Laboratory of Microbiology of the General University Hospital of Alicante (Spain) from a blood sample (hemoculture) of a neonate. *Rhizopus stolonifer* was a clinical strain from a periocular nasal fossa fat isolated by Dr. Arena (General Hospital Dr. Manuel Gea-González, Mexico DF, Mexico). All pathogenic filamentous fungi were grown on potato dextrose agar (PDA) (Becton Dickinson and Company, USA) in 9 cm Petri dishes at 25 ºC. *Neurospora crassa* used in this study, was the wild-type strain 74-OR23-IVA (FGSC #2489) kindly provided by the Fungal Genetics Stock Center (FGSC, USA). *Neurospora crassa* was grown on Vogel's solid medium (MM) (1x

Vogel's salts, 2% sucrose and 1.5% technical agar) in 9 cm Petri dishes at 25 ºC under continuous light for 5-7 days.

2.2 *Pathogenic yeasts.*

Candida albicans (SN87) was kindly provided by Dr. Nislow (University of Toronto, Canada). Reference strains for antifungal susceptibility testing were: *Candida krusei* (ATCC6258), *Candida glabrata* (ATCC2001) and *Candida parapsilosis* (ATCC22019). *Cryptococcus neoformans* (ATCC3501) and *Cryptococcus gattii* (CBS11728) were also used. Yeast were grown in 0.5x YPD broth medium pH 5 (0.5% bacto yeast extract, 1% bacto peptone and 1% dextrose) for culture proliferation and 0.5x YPD agar (1.5% bacteriological agar) was used for stock cultures.

2.3 *Chitosan.*

A medium-size molecular weight chitosan (70 kDa) with 82.5% deacetylation degree (T8s) was from Marine Bio Products GmbH (Bremerhaven, Germany). Chitosan was dissolved in 0.25 M HCl under continuous stirring and the pH of the solution was adjusted to 5.6 with 1 M NaOH as described by Palma-Guerrero et al. (2008). The resulting solution was dialyzed against distilled water for salt removal, then autoclaved at 121 ºC for 20 min and finally it was stored at 4 ºC until used.

2.4 *Effect of nutrient limitation on growth kinetics of filamentous fungi and yeast.*

Experiments in liquid media were set up to evaluate growth kinetics of *N. crassa,* other filamentous fungi and yeast human pathogens. Conidia of filamentous fungi were obtained by adding 2 ml of 0.05% Tween-20 per Petri dish, from 8-to-10 day-old sporulated cultures. The resulting conidial suspensions were then filtered through Miracloth paper (Calbiochem) to remove hyphae fragments and adjusted to a final concentration of 10^6 conidia ml^{-1} with potato dextrose broth (PDB) (Becton Dickinson).

PDB (pH 5.6) was supplemented to include 0.1, 0.5, 1, 2, 5.8 and 10 g of glucose per liter (carbon (C): 0.04; 0.2; 0.4; 0.8; 2.32; 4 g l^{-1}, respectively and nitrogen (N) contents: $1.6x10^{-3}$; $7.8x10^{-3}$; $15.5x10^{-3}$; 0.03; 0.09 and 0.15 g l^{-1}, respectively). Chitosan (0.5-1000 µg ml^{-1}) was added to these media and 200 µl per well were dispensed into 96 well microtiter plates (Sterillin Ltd., Newport, UK). Plates were inoculated with *N. crassa* conidia ($2x10^5$ conidia per well) and incubated at 25 ºC under continuous light,

without shaking. The effect of chitosan on growth of filamentous fungi was evaluated by measuring daily for five days the optical density at 490 nm (OD_{490}) (Clinical and Laboratory Standards Institute, 2008) in a GENiosTM multiwell spectrophotometer (Tecan, Männedorf, Switzerland).

Regarding pathogenic yeast, overnight cultures grown at 30ºC in 0.5x YPD broth were used for experiments. Two µl of these cultures adjusted to a final OD_{595} of 0.0625 (Burke, 2000) were added to each well of microtiter plates, containing 0-240 µg ml^{-1} of chitosan. Yeast were either grown at 30ºC (*Cryptococcus* spp.) or 37ºC (*Candida* spp.) and incubated as above and growth was spectrophotometrically monitored for 24 h. YPD was prepared by including increasing concentrations of glucose (0.1, 0.5, 1, 2, 5 and 10 g l^{-1}) as the main C source. YPD prepared with these glucose concentrations respectively included the following C (0.04; 0.2; 0.4; 0.8; 2; 4 g l^{-1}) and N (0.015; 0.074; 0.15; 0.3; 0.74 and 1.47 g l^{-1}) contents. The N content of YPD was higher than that of media for filamentous fungi (e.g. PDB), since yeast require more N for their growth than the latter. YPD was then amended with 0-240 µg ml^{-1} of chitosan. In order to evaluate the fungicidal effect of chitosan, we performed a spot assay by adding 2 µl of liquid cultures to plates with 0.5x YPD agar (1.5 % bacteriological agar) and these were incubated 48 h at 30 ºC. All assays were carried out in triplicate.

For the determination of carbon and nitrogen contents of PDB and YPD, aliquots of these nutrient media were processed in a TruSpec CN (Leco) elemental analyzer at the Common Research Facilities of the University of Alicante.

2.5 *Fungal membrane permeabilization.*

N. crassa conidia (10^6 conidia ml^{-1}) were added to media containing several concentrations of C and N (see above), and supplemented with chitosan (1-50 µg ml^{-1}). The mixture was then incubated at 25 ºC for 45 min under continuous shaking. Conidia were then stained with 30 nM Sytox Green® (Life Tech., USA) for 30 min in the dark to determine membrane permeabilization as in Thevissen et al. (1999). Fluorescence emission was evaluated in a Flow Cytometer (EPICS XL, Beckman Coulter) using 488 nm and 520 nm as an excitation and emission wavelengths, respectively. Untreated controls to determine conidia auto-fluorescence were also included. Fifteen thousand

events per sample were recorded and the data analyzed using EXPO™ 32 MultiCOMP software (Beckman Coulter). All experiments were repeated twice.

2.6 *Intracellular ROS production*.

Intracellular ROS production was estimated fluorimetrically using 2'-7' dichlorofluorescein diacetate (DCF) (Sigma, St. Louis, MO, USA). *Neurospora crassa* conidia (10^6 conidia ml^{-1}) were inoculated in media with increasing concentrations of glucose, as previously described and amended with chitosan (1-50 µg ml^{-1}). Conidia were then incubated at 25ºC under shaking (150 rpm) for 30 min. Afterwards, conidia were stained with 50 µg ml^{-1} DCF in DMSO (final concentration) and incubated for 2 h under shaking in the dark. Samples were placed in black microtiter plates (Sigma) and fluorescence was recorded with a GENiosTM multiwell reader, using a 490 nm excitation filter and a 535 nm emission filter (in triplicate). Untreated controls were incorporated to evaluate auto-fluorescence of conidia. Experiments were carried out in triplicate and all data sets obtained were checked using the Shapiro-Wilcoxon test. Data following a normal distribution were compared using ANOVA tests. The level of significance in all cases was 95%. All statistical analyses were performed with R version 2.15.1 (R Development Core Team).

2.7 *Time-course of ROS production and lethality in* N. crassa *treated with chitosan.*

Time-course experiments to evaluate ROS production in presence of chitosan were carried out under two nutritional regimes. *N. crassa* conidia (10^7 conidia ml^{-1}) were inoculated in either 2.5 g l^{-1} PDB (0.8 g l^{-1} C; 0.03 g l^{-1} N) or 12 g l^{-1} PDB (4 g l^{-1} C; 0.15 g l^{-1} N). Conidia suspensions were amended with increasing concentrations of chitosan (2.5-100 µg ml^{-1}) and then stained with DCF (see 2.6). Controls for auto-florescence of PDB and conidia were included. In addition, a positive ROS control (3% H_2O_2) was also included. All samples were dispensed into black microtiter plates and incubated (25 ºC) in the multiwell plate reader. Fluorescence of samples (see above) was recorded every 10 min for 20 h to characterize ROS induction. Lethality of chitosan treatments was estimated with the spot assay as described, using GFS agar plates (1x MM salts, 2% sorbose, 0.05% glucose, 0.05% fructose and 1.5% bacteriological agar) (Carneiro et al., 2012).

2.8 *Effect of nutrient limitation with different carbon sources on* N. crassa *sensitivity to chitosan.*

Vogel's minimal medium (MM) salts solution diluted 100 times (Vogel, 1956) was amended with 2, 5.8 and 10 g of either lactate or glucose per liter. To these media chitosan (1-50 μg ml^{-1}) was added and *N. crassa* conidia inoculated as described (see 2.4). *N. crassa* growth kinetics was evaluated spectrophotometrically as previously described. Each experiment was repeated three times.

2.9 *Chitosan cytotoxicity assay on mammalian cell lines.*

To evaluate chitosan cytotoxicity, we performed a methylthiazolyldiphenyl-tetrazolium bromide (MTT) (Sigma) colorimetric assay with human embryonic kidney HEK293 and the monkey fibroblast-like COS7 cell lines. Cells were seeded into 96-well plates, pre-coated with 10 μg ml^{-1} of poly-L-lysine for HEK293, to obtain a cellular lawn, with three replicates at $5x10^3$ and $20x10^3$ cells/well each one. Cells were grown at 37 ºC and 5% CO_2 for 24 h in Dulbecco's Modified Eagle's medium (DMEM; pH 7.5; Sigma) supplemented with 5% fetal bovine serum (FBS; Biobest), 1% L-glutamine, 1% penicillin/streptomycin (P/S; Thermo Scientific), 1% sodium pyruvate, 1% non-essential amino acids for HEK293 cells and with 10% FBS, 1% L-glutamine, 1% P/S and 1% sodium pyruvate for COS7. After incubation, cells were treated with 100 μL of DMEM supplemented with different concentrations of chitosan (1, 5, 10, 25, 50, 100 and 150 μg ml^{-1}) in each well. Cells treated with DMSO and cells in wells with no growth medium were used as positive controls for cell death. We tested the toxicity after 24 h and proliferative activity after 24 h, 48 h and 72 h of treatments at 37ºC and 5% CO_2. With this purpose, the medium was removed from wells and 100 μL of 1 mg ml^{-1} MTT was added to each well. Plates were then incubated for 4 h at 37 ºC and 5% CO_2 in the dark. The Formazan originated from MTT by the mitochondrial activity of living cells was solubilized in 100 μl of DMSO, and the absorbance at 570 nm was measured in a Benchmark Microplate Reader (Bio-Rad) (Kaushik et al., 2012; Yaris et al., 2013). All datasets obtained were analyzed using the Shapiro-Wilcoxon test. Data following a normal distribution were compared using ANOVA tests. The level of significance in all cases was 95%. All statistical analyses were performed with R version 2.15.1 (R Development Core Team). Cytotoxicity index (CI) was calculated as:

CI= (1-OD_{570} treated/OD_{570} control) x100 (Hongo et al., 1990). These experiments were carried out in triplicate.

2.10 *Inhibition and cytotoxicity evaluation of chitosan in mammalian peripheral blood lymphocyte cultures.*

Peripheral blood lymphocytes from five healthy donors were isolated by Ficoll-Hypaque (HE Healthcare) density gradient and labeled with 5 µM carboxyfluorescein diacetate succinimidyl ester (CFSE, Sigma) in 1 ml of DMEM culture medium supplemented with 10% FBS and 1% P/S for 5 min, by using a standard protocol (Lyons, 2000). Cells (10^5 per replicate) were cultured for 5 days with and without 10 µg ml^{-1} phytohemagglutinin (PHA; Sigma), it necessary to stimulate lymphocyte proliferation, under different chitosan concentrations (0, 1, 5, 7.5, 25, 50, 75, 100, 200 and 400 µg ml^{-1}). Cell proliferation was then measured by flow cytometry (as above). Prior to flow cytometry analysis, cells were also labeled with 2 µg ml^{-1} propidium iodide (PI) (Sigma) to evaluate cell death, using 488 nm and 560 nm as excitation and detection wavelengths respectively. Five thousand lymphocytes per sample (chitosan dose) were acquired to measure cell proliferation and 10,000 events were recorded to measure cell death. Data were analyzed statistically, using One-way Anova and Tukey post-hoc tests. All statistical analyses were performed with R version 2.15.1 (R Development Core Team). Experiments were carried out in triplicate.

2.11 *Effect of chitosan on* Galleria mellonella *larvae infection by* C. albicans.

Candida albicans was grown in 0.5x YPD (broth) overnight at 30 ºC with shaking. Forty eight µl of overnight cultures were diluted in either 0.25x or 0.5x YPD to obtain an inoculum with $3.1x10^6$ cells ml^{-1}. Chitosan was added to the media to reach final concentrations of 20-150 µg ml^{-1}. Twenty larvae of *G. mellonella* (provided by Animal-center S.C., Valencia, Spain) per treatment were injected (22 µl per larvae) using a 26G syringe (Terumo) as described in Fuchs et al. (2010). Larvae injected without chitosan or *C. albicans* were included as controls. Injected larvae were incubated at 30 ºC in the dark and their survival recorded daily. Mortality data were analyzed using the Kaplan-Maier survival test (Martin-Bland and Altman, 1998). All data were analyzed statistically using SPSS statistics 17.0. This experiment was carried out in triplicate.

3 RESULTS

3.1 Carbon and nitrogen limitation increase growth inhibition and membrane permeabilization caused by chitosan in *N. crassa*

The effect of chitosan on growth of *N. crassa* depended directly on the C and N content of the medium used (Figs. 1A, 1C, 1E). Nutrient limitation increased growth inhibition and membrane permeabilization caused by chitosan in *N. crassa* (Figs 1B, 1D and 1F). Under low C (0.8 g l^{-1}) and N (0.03 g l^{-1}) content, chitosan minimal inhibitory concentration (MIC) was 5 µg ml^{-1} (Fig 1A). Under this nutrient level, a very low chitosan concentration (2.5 µg ml^{-1}), caused partial membrane permeabilization and a 25% growth reduction of *N. crassa*. This chitosan dose caused a slight shift in the peak of fluorescence in cells not stained towards Sytox emission wavelength, perhaps indicating subtle membrane damage. A higher chitosan dose (3.75 µg ml^{-1}) resulted in the emission of Sytox fluorescence by all cells (Fig 1B), a fact that was associated with the disappearance of Sytox-unstained cells, indicative of massive plasma membrane permeabilization of all *N. crassa* conidia evaluated. This effect was more evident (sharper peaks) at higher concentrations of chitosan.

When C (2.32 g l^{-1}) and N (0.09 g l^{-1}) levels in the medium were raised, *N. crassa* became more tolerant to chitosan, with a MIC of 10 µg ml^{-1} (Fig 1C). Under these conditions, plasma membrane permeabilization occurred at 10 µg ml^{-1} chitosan (Fig 1D), indicating that plasma membrane was more resistant to chitosan permeabilization than at lower C and N levels. When we further increased nutrient (4 g l^{-1} of C and 0.15 g l^{-1} of N) contents, *N. crassa* stepped up its tolerance to chitosan to a MIC of 50 µg ml^{-1} (Fig 1E). Under these nutrient levels, permeabilization of *N. crassa* conidia membrane was attained at an even higher chitosan dose of 30 µg ml^{-1} (Fig. 1F). The flow cytometry profile suggested that under these conditions plasma membrane permeabilization was an “all or none” process.

Thus, nutrient (C and N) limitation enhances the chitosan antifungal effects and has a determinant role in chitosan-induced plasma membrane permeabilization in *N. crassa*. Under all nutritional statuses studied, membrane permeabilization occurred at chitosan concentrations when fungal growth was inhibited (Fig. 1).

3.2 Chitosan increases intracellular ROS production in *N. crassa*

N. crassa intracellular ROS levels increased with chitosan concentration under various nutritional regimes (Fig. 2). At low levels of C (0.8 g l^{-1}) and N (0.03 g l^{-1}), a chitosan concentration of 2.5 µg ml^{-1}, elicited a significant rise in ROS levels (Fig. 2A), which was coincident with the start of plasma membrane permeabilization (Fig. 1B). In contrast, at 5 µg ml^{-1}, ROS production did not show significant differences compared to that elicited by 2.5 µg ml^{-1} chitosan (p-value< 0.05). At intermediate C and N concentrations (2.32 and 0.09 g l^{-1}, respectively) in the medium, ROS levels raised steadily with chitosan dose, up to 5 µg ml^{-1} (Fig. 2B). Then, they remained stable up to 10 µg ml^{-1} (MIC; Fig 1C). This concentration coincided with the start of plasma membrane permeabilization (see Fig. 1D). At high nutrient (C: 4 g l^{-1} and N: 0.15 g l^{-1}) levels, a trend in ROS release similar to that found at intermediate C and N content (2.32 and 0.09 g l^{-1}, respectively) could be observed (Fig. 2C). Membrane was permeabilized with 30 µg ml^{-1} of chitosan (see Fig. 1F), after maximum ROS production was achieved (10 µg ml^{-1}). Therefore, intracellular oxidative stress seems to be an important response of *N. crassa* to chitosan.

3.3 Chitosan fungicidal activity is associated with intracellular ROS induction in *N. crassa*

A time-course study of ROS induction and mortality was carried out on *N. crassa* conidia treated with chitosan (Fig. 3). We found that under low nutrient conditions (see 3.2) chitosan at 10 µg ml^{-1} elicited a steady ROS induction (1.5-fold compared to control) which caused full mortality of *N. crassa* conidia (Fig 3A and 3B). When nutrients were raised (4 g l^{-1} C and 0.15 g l^{-1} N) chitosan above 30 µg ml^{-1} caused ROS induction (2-fold compared to control) and *N. crassa* full mortality (Fig 3C and 3D). In contrast, low chitosan concentrations had low (small spots formation) or no effect on *N. crassa* mortality at the two nutrient regimes tested. Control conidia displayed a ROS induction similar to that found at low chitosan doses.

3.4 Chitosan exhibits greater antifungal activity to *N. crassa* with lactate instead of glucose as main carbon source

A nutrient (C and N) reduction when lactate replaced glucose as the main carbon source enhanced chitosan antifungal activity in *N. crassa* (Fig. 4). At low concentrations of C and N when 2 g l^{-1} glucose was used as the main C source (C, 0.8 g l^{-1}; N, 0.0014 g l^{-1}),

N. crassa was less sensitive to chitosan (MIC 10 µg ml^{-1}, Fig. 4A), than when an equivalent concentration of lactate was used instead of glucose (MIC, 2.5 µg ml^{-1}, Fig. 4B). On the other hand, intermediate concentrations of C and N (5.8 g l^{-1}glucose) (C, 2.32 g l^{-1}; N, 0.004 g l^{-1}) elicited no changes in *N. crassa* sensitivity to chitosan (MIC 10 µg ml^{-1}, Fig. 4C). Replacing glucose by lactate resulted in an increase of *N. crassa* sensitivity to chitosan (MIC 2.5 µg ml^{-1}, Fig. 4D). Although increasing nutrient content when lactate was used as the main C source did not change *N. crassa* sensitivity to chitosan (MIC 2.5 µg ml^{-1}), growth of this fungus increased by ca. two-fold with respect to that at 2 g l^{-1} of lactate (Fig. 4B and 4D). An increase in C and N levels (C, 4 g l^{-1}; N, 0.007 g l^{-1}) in the medium (10 g l^{-1} glucose) diminished *N. crassa* sensitivity to chitosan (MIC 17.5 µg ml^{-1}) (Fig. 4E) and under these conditions, glucose replacement by lactate increased chitosan sensitivity of the fungus (MIC 5 µg ml^{-1}) (Fig. 4F). These results indicate that when lactate was used instead of glucose as the main carbon source, chitosan antifungal activity on *N. crassa* was enhanced by ca. 4 times.

3.5 Carbon and nitrogen limitation increase chitosan susceptibility in filamentous fungi and yeast human pathogens

In view of our previous results on the enhancement of chitosan antifungal effect by nutrient (C and N) limitation on *N. crassa*, we performed similar tests on a wide variety of clinically important human fungal pathogens (Fig. 5). Carbon (0.4 g l^{-1}) and nitrogen (0.016 g l^{-1}) concentrations in the growth medium were set to normal value of human blood glucose content (glycemia). At these nutrient contents, chitosan (2.5 µg ml^{-1}) caused a moderate (ca. 25%) growth inhibition in *F. proliferatum* with MIC and minimal fungicidal concentration (MFC) of 5 µg ml^{-1} (Fig. 5A, Table 1). Under the same C and N contents, chitosan (above 40 µg ml^{-1}) caused growth inhibition on *A. fumigatus* during the first 48 h, but later on (after 72 h) the fungus resumed growth (Table 1 and Suppl. Fig 1L). At the same C level and with 0.15 g l^{-1} of N, chitosan MIC was 10 µg ml^{-1} for *C. albicans* (Fig. 5D) and 20 µg ml^{-1} for *C. krusei* (fluconazole resistant strain, Suppl. Fig. 2F) and *C. neoformans* (Suppl. Fig. 2P). MFC for *C. albicans* and *C. krusei* was 20 µg ml^{-1} (Table 1).

A reduction of carbon (0.2 g l^{-1}) (similar to low levels of glucose in blood, hypoglycemia) and nitrogen (0.074 g l^{-1}) in the culture medium increased the chitosan

antifungal effects (Table 1). For instance, for *C. albicans* chitosan MIC was again 10 µg ml^{-1}, but MFC became reduced (10 µg ml^{-1}) with respect to the value found at higher nutrient content. At severe C (0.04 g l^{-1}) and N (0.015 g l^{-1}) limitation chitosan MIC was further reduced to 5 µg ml^{-1} (Table 1). An increase in sensitivity to chitosan with nutrient limitation was also found for the rest of fungal human pathogens tested (Table 1).

Conversely, high levels of C and N reduced chitosan antifungal activity on the fungi (Fig. 5, Table 1 and Suppl. Figs. 1, 2). For instance, *C. albicans* growing from 0.8 g l^{-1} of C and 0.3 of N to 4 g l^{-1} of C and 1.5 g l^{-1} of N, increased chitosan MIC from 20 to 80 µg ml^{-1} (Table 1) . The fungicidal activity of chitosan at low levels of nutrients increased respect to that at high nutrient content in *Candida* spp. (Table 1, Fig. 6). Furthermore, other *Candida* spp. (*C. krusei*, *C. glabrata* and *C. parapsilosis*) also proved to be sensitive to chitosan (Table 1, Suppl. Fig. 2). This pattern was also found in *C. neoformans* and *C. gattii* (Table 1, Suppl. Fig. 2), both yeasts showed less chitosan antifungal sensitivity at high than at low nutritional (C and N) levels. Regarding filamentous fungal pathogens, *F. proliferatum* (Table 1) and *H. avellanea* (Suppl. Fig. 1) were sensitive to chitosan and their MICs increased from 7.5 and 5 µg ml^{-1}, from 0.8 g l^{-1} C and 0.03 g l^{-1} N to 40 and 30 µg ml^{-1} at 4 g l^{-1} C and 0.15 g l^{-1} N, respectively (Table 1). On the contrary, *A. fumigatus* and *R. stolonifer* were chitosan insensitive at all these high C and N levels. These fungi reduced its growth at high concentration of chitosan, but this compound never was fungicide for both species.

3.6 Chitosan does not affect growth of HEK293 and COS7 cell lines

Chitosan was tested on two mammalian cell lines (HEK293 and COS7; Fig. 7). No effect on cell viability was found after 24 h (Fig. 7A and 7B). At the two cell densities tested, chitosan at 1-150 µg ml^{-1} displayed no differences on cell viability ($P < 0.05$) with respect to untreated controls. On the contrary, virtually full mortality was found when cells were either dried (physical disturbance) or treated with DMSO (chemical disturbance). The cytotoxicity index (CI) of chitosan did not exceed 50% of inhibitory concentration (IC_{50}) (Suppl. Table 1).

Chitosan cytotoxicity was evaluated over time in order to determine its effect on cell proliferation. Chitosan showed no effect on cell proliferation at 48 h (Figs. 7C and 7D)

for all concentrations tested, except at 150 µg ml^{-1}. At this concentration, COS7 (CI value 88 at 72 h) but especially HEK293 cells, were sensitive to chitosan (CI value, 61 at 48h) (Suppl. Table 2).

3.7 Chitosan has a low effect on human lymphocytes

The effect of chitosan on mortality and proliferation of human lymphocytes was evaluated (Fig. 8). Chitosan cytotoxicity on human lymphocytes never reached values higher than 20% (7.5 µg ml^{-1}) of that found in untreated control cells (Fig. 8A). No clear correlation between chitosan concentrations and cytotoxicity was observed. For instance, with the high range of chitosan concentration tested (25-400 µg ml^{-1}), only 50 and 400 µg ml^{-1} had an effect on cell death, but cells treated with chitosan at 75-200 µg ml^{-1} showed no significantly differences in their viability compared to controls (Fig. 8A).

A slight reduction in lymphocyte proliferation (IC_{30}, 28%) was found when chitosan was added at 25 µg ml^{-1} (Fig. 8B). However, differences in lymphocyte proliferation in cells treated with chitosan were found not statistically different respect to controls (without chitosan).

3.8 Nutrient limitation promotes chitosan reduction of *C. albicans* virulence to *G. mellonella*

Chitosan significantly reduced *C. albicans* virulence on larvae of the moth *G. mellonella* measured as cumulative survival (Fig. 9). *C. albicans* caused high larvae mortality at 2 g l^{-1} C and 0.74 g l^{-1} N in the medium, since all infected larvae without chitosan (positive controls) died within 24 h (Fig. 9B). Interestingly, chitosan increased *G. mellonella* larvae survival in a concentration-dependent fashion under these conditions. After 48 h, ca. 20% of the larvae treated with 20 µg ml^{-1} of chitosan survived and at higher concentrations of chitosan (100 µg ml^{-1}) larval survival was close to 100%, the mortality confidence interval (152-262 h) showed significant differences ($p < 0.05$) compared to that of controls.

When nutrient content was increased (C, 4 g l^{-1} and N, 0.15 g l^{-1}) *C. albicans* virulence on *G. mellonella* was reduced (Fig. 9D). Therefore, the effect of chitosan on *C. albicans* virulence was less evident than that at low concentrations of nutrients. After 48 h, a 65%

survival was achieved at 100 µg ml^{-1} compared to 40% survival at 40 µg ml^{-1} chitosan. Under different nutrient regimes, non-injected larvae exhibited mortalities similar to those of controls when they were injected with chitosan only and no *C. albicans* (Fig. 9A and 9C).

4 DISCUSSION

We here demonstrated that nutrient (C and N) limitation increased *N. crassa* sensitivity to chitosan. Some features associated with fungal nutrient deprivation (hyphal fragmentation, ammonium release and increase in extracellular hydrolytic activities) (Nitsche et al., 2012; Szilágyi et al., 2013) could modify *N. crassa* cell wall architecture making it more permeable to chitosan. Under these conditions chitosan could then reach more easily the plasma membrane, facilitating its permeabilization. Alternatively, C and N limitation could result in lack of energy necessary for the cell wall and for plasma membrane repair after chitosan damage.

We have been able to link the increase in chitosan sensitivity due to C and N limitation to plasma membrane permeabilization and intracellular ROS production. This finding, reported for the first time, would explain why sodium azide, which uncouples oxidative metabolism and prevents ROS production, protects *N. crassa* from chitosan by preventing membrane permeabilization (Palma-Guerrero et al., 2009; Carneiro et al., 2012). In the present work, we also demonstrate that chitosan fungicidal activity to which *N. crassa* is sensitive is associated with the induction of intracellular ROS by this fungus (a chitosan sensitive fungus). ROS could cause membrane permeabilization by means of lipid peroxidation, mostly to polyunsaturated fatty acids (Howlett and Avery, 1997), which are very abundant in *N. crassa* and other chitosan-sensitive fungi (Palma-Guerrero et al., 2010). At chitosan concentrations where complete membrane permeabilization took place, ROS production decreased. Mitochondrial membrane permeabilization would cause uncoupling of the electron transport chain, the main source of intracellular ROS (Kowaltowski et al., 2009; Carneiro et al., 2012), this explaining our experimental results. ROS production is a by-product from processes such as programmed cell death, abiotic stress and systemic signaling in filamentous fungi and plants (Mittler, 2002; Aguirre et al., 2005; Mittler et al., 2011). ROS have also been associated with the mode of action of currently used antifungals such as

miconazole (Ene et al., 2012a). We have found in this work that when *N. crassa* was released from C and N limitation, chitosan antifungal activity was reduced. High nutrient supply would increase metabolic rate and generate ROS as by-product. However, at the same time a high amount of antioxidant enzymes could be synthesized in order to scavenge ROS excess and prevent cell damage (Yan et al., 2006). An alternative way to reduce the hyperoxidant state in fungi treated with chitosan would be to increase reducing power by supplying a higher C and N content to the growth medium. In this regard, when lactate was used as a main carbon source instead of glucose, C and N limitation increased *N. crassa* sensitivity to chitosan to a larger extent (ca. 4-fold) compared to glucose. Since chitosan antifungal activity depends directly on the energetic status of the cell, our result could be explained because lactate is able to provide less reducing power and it is less efficient energetically than glucose.

The nutritional status also affected the sensitivity of filamentous fungi and yeast pathogens to chitosan. This increase in sensitivity with nutrient limitation has also been found in *C. albicans* for other antifungals (Ene et al., 2012a; Ene et al., 2012b). *Candida* spp. have shown a high sensitivity to chitosan in previous studies (Peña et al., 2013; Kulikov et al., 2014). *F. proliferatum* is an important human pathogen which causes mycoses in immunosuppressed patients (Summerbell et al., 1988) which was found in our study even more sensitive to chitosan than *Candida* spp. The plant pathogen *F. oxysporum* f. sp. *radicis-lycopersici* was previously found to be highly sensitive to chitosan (Palma-Guerrero et al., 2008). We have also found *Cryptococcus* spp. to be sensitive to chitosan. This polymer has been found to be a cell wall component of *C. neoformans* and a requirement for virulence and persistence in mammalian hosts of the fungus (Baker et al., 2007; Baker et al., 2011). Chitosan added exogenously to the growth medium could generate a chitosan excess which would determine its antifungal effect to *Cryptococcus* spp. found in our work. As for the other human pathogens, the C and N status influence in the same direction chitosan antifungal activity on *C. neoformans* and *C. gattii.*

Most chitosan concentrations tested in our study were non-toxic for mammalian cells (Qi et al., 2005). We found a low cytotoxicity of chitosan on lymphocytes, but no effect on their proliferation as found in other studies (Borges et al., 2007). In our study, we have shown that chitosan reduced *C. albicans* virulence on *G. mellonella*. This

experimental insect host has been previously used to evaluate the effect of other antimicrobials on *Candida* spp. virulence (Cowen et al., 2009; Mesa-Arango et al., 2013). Besides, we have also reported that lowering nutrients increased this inhibitory effect of chitosan on *C. albicans* virulence. Future studies should aim to investigate the basis of the reduction of virulence and the increase of antifungal effect of chitosan associated with C and N limitation, because *C. albicans* encounters carbon-poor conditions during infection and growth in its hosts (Lorenz and Fink, 2001). To this respect, alternative carbon sources are known to strongly influence *C. albicans* virulence and its susceptibility to antifungal drugs (Ene et al., 2012a).

In conclusion, we have shown that nutrient (C and N) status and ROS are key factors of chitosan antifungal mode of action. The mechanisms by which both factors cause fungal membrane permeabilization and death should be further investigated. To this respect, *N. crassa,* high levels of ROS are known to activate the mechanisms which trigger programmed cell death (Castro et al., 2008). The liaison between ROS production, membrane permeabilization and its implication in membrane lipid peroxidation should be established by evaluating this process under diverse nutrient (C and N) conditions and chitosan doses. Other alternative mechanisms by which chitosan permeabilizes membranes and kills fungal cells cannot be excluded. Perhaps a balance between ROS detoxification and antioxidant mechanisms could be the key to understand the differential sensitivity of fungi to chitosan. Future studies should also contemplate the effect of ROS scavenging nutrients (Chen and Dickman, 2005) and the expression of ROS detoxification enzymes on chitosan antifungal activity. Our results illustrate that chitosan is a promising antifungal agent towards relevant human fungal pathogens whose potency can be enhanced by modifying the nutritional status in its environment. The low toxicity on mammalian cells opens new opportunities for the clinical use of chitosan as an antifungal alone or in combination with conventional antifungals.

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REFERENCES

Aguirre J., Ríos-Momberg M., Hewitt D., Hansberg W., 2005. Reactive oxygen species and development in microbial eukaryotes. Trends Microbiol. **13** (3), 111-118.

Allan, C.R., Hadwiger L.A., 1979. The fungicidal effect of chitosan on fungi of varying cell wall composition. Exp. Mycol. **3**, 285-287.

Baker L.G., Specht C.A., Donlin M.J., Lodge J.K., 2007. Chitosan, the deacetylated for of the chitin, is necessary for cell wall integrity in *Cryptococcus neoformans*. Eukaryot. Cell **6** (5), 855-867.

Baker, L. G., Specht, C. A., Lodge, J. K., 2011. Cell wall chitosan is necessary for virulence in the opportunistic pathogen *Cryptococcus neoformans*. Eukaryot. Cell **10** (9), 1264-1268.

Borges O., Borchard G., de Sousa A., Junginger H.E., Cordeiro-da-Silva A., 2007. Induction of lymphocytes activated marker CD69 following exposure to chitosan and alginate biopolymers. Int. J. Pharm. **337** (1-2), 254-264.

Burke D., 2000. Methods in yeast genetics: a Cold Spring Harbor Laboratory course manual. Plainview, N.Y., Cold Spring Harbor Laboratory.

Calamari, S., Bojanich, M., Barembaum, S., Berdicevski, N., Azcurra, A., 2011. Antifungal and post-antifungal effects of chlorhexidine, fluconazole, chitosan and its combinations on *Candida albicans*. Med. Oral., Pat. Oral & Cir. Bucal **16** (1), e23-e28.

Carneiro P., Duarte M., Videira A., 2012. Disruption of alternative NAD(P)H dehydrogenases leads to decreased mitochondrial ROS in *Neurospora crassa*. Free Radic. Biol. Med. **52** (2), 402-409.

Castro A., Lemos C., Falcão A., Glass N.L., Videira A., 2008. Increased resistance of complex I mutants to phytosphingosine-induced programmed cell death. J. Biol. Chem. **283** (28), 19314-19321.

Chen C. Dickman M.B., 2005. Proline suppresses apoptosis in the fungal pathogen *Colletotrichum trifolii*. Proc. Natl. Acad. Sci. USA **102** (9), 3459-3464.

Clinical and Laboratory Standards Institute, 2008. Reference method for broth dilution antifungals susceptibility testing of filamentous fungi; approved standards; 2nd edition. CLSI Document M38-A2. Clinical and Laboratory Standards Institute. Clinical and Laboratory Standards Institute, Wayne, Pa.

Cowen L.E., Singh S.D., Köhler J.R., Collins C., Zaas A.K., Schell W.A., Aziz H., Mylonakis E., Perfect J.R., Whitesell L., Lindquist S., 2009. Harnessing Hsp90 function as a powerful, broadly effective therapeutic strategy for fungal infectious disease. Proc. Natl. Acad. Sci. USA **106** (8), 2818-2823.

Ene I.V., Adya A.K., Wehmeier S., Brand A.C., Maccallum D.M., Gow N.A.R., Brown A.J.P., 2012a. Host carbon sources modulate cell wall architecture, drug resistance and virulence in a fungal pathogen. Cell. Microbiol. **14** (9), 1319-1335.

Ene I.V., Heilmann C.J., Sorgo A.G., Walker L.A., De Koster C.G., Munro C.A., Klis F.M., Brown, A.J.P., 2012b. Carbon source-induced reprogramming of the cell wall proteome and secretome modulates the adherence and drug resistance of the fungal pathogen *Candida albicans*. Proteomics **12** (21), 3164-3179.

Fuchs B.B., O'Brien E., Khoury J.B.E., Mylonakis E., 2010. Methods for using *Galleria mellonella* as a model host to study fungal pathogenesis. Virulence **1** (6), pp. 475-482.

Hongo T., Fujii Y., Igarashi Y., 1990. An in vitro chemosensitivity test for the screening of anti-cancer drugs in childhood leukemia. Cancer **65** (6), 1263-72.

Howlett N.G., Avery S.V., 1997. Induction of lipid peroxidation during heavy metal stress in *Saccharomyces cerevisiae* and influence on plasma membrane fatty acid unsaturation. Appl. Environ. Microbiol. **63** (8) 2971-2976.

Jaime M.D.L.A., Lopez-Llorca L.V., Conesa A., Lee A.Y., Proctor M., Heisler L.E., Gebbia M., Giaever G., Westwood J.T., Nislow C., 2012. Identification of yeast genes that confer resistance to chitosan oligosaccharide (COS) using chemogenomics. BMC Genomics **13** (1), 267.

Kaushik N.K., Kim H.S., Chae Y.J., Lee Y.N., Kwon G., Choi E.H., Kim I.T., 2012. Synthesis and anticancer activity of di (3-thienyl) methanol and di (3-thienyl) methane. Molecules **17** (10), 11456-11468.

583 Kowaltowski A.J., de Souza-Pinto N.C., Castilho R.F., Vercesi A.E., 2009.
584 Mitochondria and reactive oxygen species. Free Radic. Biol. Med. **47**, 333–343.
585 Kulikov, S. N., Lisovskaya, S. A., Zelenikhin, P. V., Bezrodnykh, E. A., Shakirova, D.
586 R., Blagodatskikh, I. V., & Tikhonov, V. E., 2014. Antifungal activity of
587 oligochitosans (short chain chitosans) against some candida species and clinical
588 isolates of *Candida albicans*: Molecular weight-activity relationship. Eur. J.
589 Med. Chem. **74**, 169-178.
590 Kumar, R.M.N.V., 2000. A review of chitin and chitosan applications. React. Funct.
591 Polym. **46** (1), 1-27.
592 Lorenz M.C., Fink G.R., 2001. The glyoxylate cycle is required for fungal virulence.
593 Nature **412** (6842), 83-86.
594 Lyons A.B., 2000. Analysing cell division in vivo and in vitro using flow cytometric
595 measurement of CFSE dye dilution. J. Immunol. Methods **243** (1-2), 147-154.
596 Martin-Bland J., Altman D.G., 1998. Survival probabilities (the Kaplan-Meier method).
597 BMJ **317** (7172), 1572.
598 Mesa-Arango A.C., Forastiero A., Bernal-Martínez L., Cuenca-Estrella M., Mellado E.,
599 Zaragoza O., 2013. The non-mammalian host *Galleria mellonella* can be used
600 to study the virulence of the fungal pathogen *Candida tropicalis* and the
601 efficacy of antifungal drugs during infection by this pathogenic yeast. Med.
602 Mycol. **51** (5), 461-472.
603 Miceli M.H., Díaz J.A., Lee, S.A., 2011. Emerging opportunistic yeast
604 infections. Lancet Infect. Dis. **11** (2), 142-151
605 Mittler R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. **7**
606 (9), 405-410.
607 Mittler R., Vanderauwera S., Suzuki N., Miller G., Tognetti V.B., Vandepoele K.,
608 Gollery M., Shulaev V., Van Breusegem F., 2011. ROS signaling: The new
609 wave? Trends Plant Sci. **16** (6), 300-309.
610 Nitsche B.M., Jørgensen T.R., Akeroyd M., Meyer V., Ram A.F.J., 2012. The carbon
611 starvation response of *Aspergillus niger* during submerged cultivation: Insights
612 from the transcriptome and secretome. BMC Genomics **13** (1), 380.
613 Palma-Guerrero J., Jansson H-B., Salinas J., Lopez-Llorca L.V., 2008. Effect of
614 chitosan on hyphal growth and spore germination of plant pathogenic and
615 biocontrol fungi. J. Appl. Microbiol. **104** (2), 541-553.

Palma-Guerrero J., Huang I. C., Jansson H-B., Salinas J., Lopez-Llorca L.V., Read N.D., 2009. Chitosan permeabilizes the plasma membrane and kills cells of *Neurospora crassa* in an energy dependent manner. Fungal Genet. Biol. **46**, 585–594.

Palma-Guerrero J., Lopez-Jimenez J.A., Pérez-Berná A.J., Huang I., Jansson H., Salinas J., Lopez-Llorca L.V., 2010. Membrane fluidity determines sensitivity of filamentous fungi to chitosan. Mol. Microbiol. **75** (4), 1021-1032.

Peña, A., Sánchez, N. S., Calahorra, M., 2013. Effects of chitosan on *Candida albicans*: Conditions for its antifungal activity. Biomed Res Int. 2013.

Prigneau O., Porta A., Poudrier J.A., Colonna-Romano S., Noël T., Maresca B., 2003. Genes involve in β-oxidation, energy metabolism and glyoxylate cycle are induced by *Candida albicans* during macrophage infection. Yeast **20** (8), 723-730.

Qi L., Xu Z., Jiang X., Li Y., Wang M., 2005. Cytotoxic activities of chitosan nanoparticles and copper-loaded nanoparticles. Bioorg. Med. Chem. Lett. **15** (5), 1397-1399.

Ramírez M.A., Lorenz M.C., 2007. Mutations in alternative carbon utilization pathways in Candida albicans attenuate virulence and confer pleiotropic phenotypes. Eukaryot. Cell **6** (2), 280-290.

Richardson M., Lass-Flörl C., 2008. Changing epidemiology of systemic fungal infections. Clin. Microbiol. Infect. **14** (4), 5-24.

Shapiro R.S., Robbins N., Cowen L.E., 2011. Regulatory circuitry governing fungal development, drug resistance, and disease. Microbiol. Mol. Biol. Rev. **75** (2), 213-267.

Summerbell R.C., Richardson S.E., Kane J., 1988. *Fusarium proliferatum* as an agent of disseminated infection in an immunosuppressed patient. J. Clin. Microbiol. **26** (1), 82-87.

Szilágyi M., Miskei M., Karányi Z., Lenkey B., Pócsi I., Emri T., 2013. Transcriptome changes initiated by carbon starvation in *Aspergillus nidulans*. Microbiology (UK). **159** (1), 176-190.

Thevissen K., Terras F.R., Broekaert W.F., 1999. Permeabilization of fungal membranes by plant defensins inhibits fungal growth. Appl. Environ. Microbiol. **65**, 5451–5458.

649 Vogel, H. J., 1956. A convenient growth medium for *Neurospora*. Microb. Genet. Bull.
650 **13**, 42–46.
651 Yan Y., Wei C., Zhang W., Cheng H., Liu J., 2006. Cross-talk between calcium and
652 reactive oxygen species signaling. Acta Pharmacol. Sin. **27** (7), 821-826.
653 Yari S., Parivar K., Nabiuni M., Keramatipour M., 2013. Effect of embryonic
654 cerebrospinal fluid on proliferation and differentiation of neuroprogenitor
655 cells. Cell J. **15** (1), 29-36
656 Younes, I., Sellimi, S., Rinaudo, M., Jellouli, K., Nasri, M., 2014. Influence of
657 acetylation degree and molecular weight of homogeneous chitosans on
658 antibacterial and antifungal activities. Int. J. Food Microbiol. **185**, 57-63.
659 Zakrzewska A., Boorsma A., Brul S., Hellingwerf K.J., Klis F.M., 2005. Transcriptional
660 response of *Saccharomyces cerevisiae* to the plasma membrane-perturbing
661 compound chitosan. Eukaryot. Cell **4**, 703–715.

662 **FIGURE LEGENDS**

663 **Figure 1.** Effect of nutrient (C and N) content on chitosan antifungal activity on
664 growth and membrane permeabilization in *N. crassa*. (**A, C** and **E**) show the effect
665 of chitosan on growth kinetics and (**B, D** and **F**), the evaluation of cell membrane
666 permeabilization by Sytox Green© in *N. crassa*, under increasing nutrient (C and N)
667 content of the culture medium: 0.8 C; 0.03 N g l^{-1} (**A, B**), 2.32 C; 0.09 N g l^{-1} (**C, D**)
668 and 4 C; 0.15 N g l^{-1} (**E, F**), respectively. Chitosan concentrations used are shown in
669 each graph. (**A, C** and **E**) graph values are the average of 4 estimations with their
670 standard error (SE). (**B, D** and **F**) graphs represent the accumulated values from 15
671 000 events per each single curve.

672 **Figure 2.** Intracellular reactive oxygen species (ROS) production by *N. crassa*
673 conidia treated with chitosan under different nutrient (C and N) statuses. ROS
674 production was determined by DCF fluorescence with increasing chitosan
675 concentrations (0-50 µg ml^{-1}) under 3 nutritional (C and N) regimes: (**A**) 0.8 C; 0.03
676 N g l^{-1} (**B**), 2.32 C; 0.09 N g l^{-1} and (**C**) 4 C; 0.15 N g l^{-1}. Different letters indicate
677 significant differences ($P < 0.05$). Values are the average of 4 estimations with their
678 standard error (SE).

679 **Figure 3.** Chitosan elicitation of ROS production and fungicidal activity in *N.*
680 *crassa* conidia under two nutrient (C and N) statuses. (**A, B**) C and N low content
681 (0.8 C; 0.03 N g l^{-1}) and (**C, D**) C and N high content (4 C; 0.15 N g l^{-1}) media. (**A,**
682 **C**) ROS induction. (**B, D**) Serial dilutions (from left to right) of conidial suspensions
683 of *N. crassa* wild-type strain were spotted and incubated on GFS agar plates at 25ºC
684 for 48h. Values are the average of 4 estimations as above.

685 **Figure 4.** Effect of carbon source (lactate vs. glucose) on sensitivity of *N. crassa* to
686 chitosan under various nutrient (C and N) regimes. (**A, C, E**) 2, 5.8 and 10 g l^{-1}
687 glucose, respectively, in 1/100 Vogel's salts solution. (**B, D, F**) 2, 5.8 and 10 g l^{-1}
688 lactate, respectively, in 1/100 Vogel's salts solution. Nutrient (C and N) content of
689 culture media: (**A, B**) 0.8 C; 0.0014 N g l^{-1}, (**C, D**) 2.32 C; 0.004 N g l^{-1} and (**E, F**) 4
690 C; 0.007 N g l^{-1}. Values are the average of 4 estimations as above.

691 **Figure 5.** Effect of chitosan on growth kinetics of relevant filamentous fungi and
692 yeast pathogens under different nutritional regimes. (**A, B, C**) *Fusarium*
693 *proliferatum,* (**D, E, F**) and *Candida albicans*. Nutrient (C and N) contents of
694 medium for *F. proliferatum* were*:* (**A**) 0.4 C; 0.016 N g l^{-1}, (**B**) 0.8 C; 0.03 N g l^{-1}
695 and (**C**) 4 C; 0.15 N g l^{-1}. For *C. albicans*: (**D**) 0.4 C; 0.15 N g l^{-1}, (**E**) 0.8 C; 0.3 N g
696 l^{-1} and (**F**) 4 C; 1.5 N g l^{-1}. Values are the average of 4 estimations with their
697 standard error (SE).

698 **Figure 6.** Chitosan susceptibility profiles of *C. albicans* determined by spot assay.
699 *C. albicans* cells were cultured on YPD agar plates. (**A**) YPD 0.25x (0.8-0.3 g l^{-1} C
700 and N content), (**B**) YPD 0.5x (4 C; 1.5 N g L^{-1}, C and N contents). Aliquots of 2 µl
701 from 24 h cell cultures supplemented with chitosan were diluted serially (10-fold)
702 and spotted on the YPD plates with chitosan (0-150 µg ml^{-1}). Growth differences
703 were detected after 48 h incubation at 30 ºC.

704 **Figure 7.** Chitosan effects on viability and proliferation of mammalian cell lines.
705 (**A, C**) HEK293 and (**B, D**) COS7 human and monkey cell lines, respectively. (**A,**
706 **B**) Cytotoxic effect of chitosan determined at 24 h. (**C, D**) Effect of chitosan on cell
707 proliferation at 24, 48 and 72 h. Both parameters were measured using the MTT
708 assay (OD_{570}). Different letters (a-d) indicate significant differences ($P < 0.05$).

709 Statistical analyses were carried out independently for each experiment (eg. number
710 of cells for cell viability or time for cell proliferation).

711 **Figure 8.** Effect of chitosan on human lymphocytes. (**A**) Lymphocyte mortality
712 estimated by propidium iodide staining after flow cytometry. (**B**) Lymphocyte
713 proliferation determined with the CFSE cell proliferation kit after flow cytometry.
714 Asterisks indicate significant differences ($P< 0.05$) in lymphocyte mortality. Line
715 indicates the half maximal inhibitory concentration (IC_{50}) for chitosan. Values are
716 the average ±SE from 6 estimations made on independent lymphocyte populations.

717 **Figure 9.** Effect of chitosan on virulence of *Candida albicans* on *Galleria*
718 *mellonella* under different nutrient (C and N) regimes in YPD medium. (**A**) and (**C**)
719 YPD 0.25x (0.8 C; 0.3 N g l^{-1}) and YPD 0.5x (4 C; 1.5 N g l^{-1}) respectively without
720 *C. albicans* (mortality by chitosan). (**B**) and (**D**) YPD 0.25x (0.8 C; 0.3 N g l^{-1}) and
721 YPD 0.5x (4 C; 1.5 N g l^{-1}) respectively, inoculated with *C. albicans.* Mortality
722 confidence interval 152-262 h for 100 µg ml^{-1} of chitosan ($p< 0.05$). Treatments:
723 Controls= no chitosan; chi= chitosan concentrations. N/P= *Galleria mellonella*
724 larvae non-injected (no treatment) at all.

725

726 **Table 1.** Antifungal effect of chitosan on important species of filamentous fungi and
727 yeast human pathogens under different nutrient (C and N) conditions. Minimal
728 inhibitory concentration (MIC; µg ml^{-1}) and minimal fungicidal concentration
729 (MFC; µg ml^{-1}) for chitosan on the fungal pathogens species are given.

730

731 **Supplementary Figure 1.** Effect of chitosan on growth of filamentous fungal
732 human pathogens under different nutrient (C and N) conditions. (**A, B, C**) *Hamigera*
733 *avellanea* and (**D, E, F**) *Rhizopus stolonifer.* C and N contents of the medium were:
734 (**A, D**) 0.8 C; 0.031 N g l^{-1}, (**B, E**) 2.32 C; 0.087 N g l^{-1}, (**C, F**) 4 C; 0.15 N g l^{-1},
735 respectively. (**G, H, I**) *Fusarium proliferatum.* C and N contents of the medium
736 were: (**G**) 0.04 C; 0.0015 N g l^{-1}, (**H**) 0.2 C; 0.007 N g l^{-1}, (**I**) 4 C 0.15; N g l^{-1} and
737 (**J-O**) *Aspergillus fumigatus.* C and N contents of the medium were: (**J**) 0.04 C;

738 0.0015 N g l^{-1}, (**K**) 0.2 C; 0.007 N g l^{-1}, (**L**) 0.4 C; 0.015 N g l^{-1}, (**M**) 0.8 C; 0.031 N
739 g l^{-1}, (**N**) 2.32 C; 0.087 N g l^{-1}, (**O**) 4 C; 0.15 N g l^{-1}, respectively.

740 **Supplementary Figure 2.** Chitosan effect on growth of yeast human pathogens
741 under different nutrient (C and N) conditions. (**A, B, C**) *Candida albicans.* C and N
742 contents of the medium were: (**A**) 0.04 C; 0.015 N g l^{-1}, (**B**) 0.2 C; 0.074 N g l^{-1}, (**C**)
743 2 C; 0.74 N g l^{-1}, respectively. (**D, E, F, G, H, I**) *Candida krusei.* C and N contents
744 of the medium were: (**D**) 0.04 C; 0.015 N g l^{-1}, (**E**) 0.2 C; 0.074 N g l^{-1}, (**F**) 0.4 C;
745 0.15 N g l^{-1},(**G**) 0.8 C; 0.3 N g l^{-1}, (**H**) 2 C; 0.74 N g l^{-1}, (**I**) 4 C 1.5 N g l^{-1},
746 respectively. (**J, K**) *Candida glabrata* and (**L, M**) *Candida parapsilosis.* C and N
747 contents of the medium were: (**J, L**) 2 C; 0.74 N g l^{-1} and (**K, M**) 4 C; 1.5 N g l^{-1}.
748 (**N-U**) *Cryptococcus* spp.: *Cryptococcus neoformans.* C and N contents of the
749 medium were: (**N**) 0.04 C; 0.015 N g l^{-1}, (O) 0.2 C; 0.074 N g l^{-1}, (**P**) 0.4 C; 0.15 N
750 g l^{-1},(**Q**) 0.8 C; 0.3 N g l^{-1}, (**R**) 2 C; 0.74 N g l^{-1}, (**S**) 4 C; 1.5 N g l^{-1} and
751 *Cryptococcus gattii*. C and N contents of medium were: (**T**) 2 C; 0.74 N g l^{-1}, (**U**) 4
752 C; 1.5 N g l^{-1}.

753

754 **Supplementary Table 1.** Effect of chitosan on human HEK293 and monkey COS7
755 cell lines. The effect was estimated using a cytotoxic index defined as (1-$OD_{470treated}$-
756 $OD_{470controls}$)x100.

757 **Supplementary Table 2.** Effect of chitosan on proliferation of human HEK293 and
758 monkey COS7 cell lines. The effect was estimated using a cytotoxic index defined
759 as (1- $OD_{470treated}$-$OD_{470controls}$)x100.

Table 1. Antifungal effect of chitosan on important species of filamentous fungi and yeast human pathogens under different nutrient (C and N) conditions. Minimal inhibitory concentration (MIC; μg ml-1) and minimal fungicidal concentration (MFC; μg ml-1) for chitosan on the fungal pathogens species are given.

[C and N] (g L^{-1})	**0.04/0.0015¥**			**0.2/0.007¥**			**0.4/0.015¥**			**0.8/0.031¥**			**2.32/0.087¥**			**4/0.15¥**		
Filamentous fungi	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Fusarium proliferatum	2.5	2.5	2.5*	2.5	2.5	2.5*	2.5	5	5*	5	7.5	7.5*	10	20	20*	20	30	40*
Hamigera avellanea										2.5	5	5*	10	20	20*	10	20	30*
Aspergillus fumigatus	1	1	1000	1	5	1000	1	40	1000	100	1000	1000	100	ni	ni	1000	ni	ni
Rhizopus stolonifer										100	ni	ni	ni	ni	ni	ni	ni	ni
[C and N] (g L^{-1})	**0.04/0.015¥**			**0.2/0.074¥**			**0.4/0.15¥**			**0.8/0.3¥**			**2/0.74¥**			**4/1.5¥**		
Yeast	6 h	12 h	24 h	6 h	12 h	24 h	6 h	12h	24h	6h	12h	24h	6h	12h	24h	6h	12h	24h
Candida albicans	5	5	5 (10)	5	10	10*	10	10	10 (20)	20	20	20 (40)	20	40	40 (80)	40	80	80 (100)
Candida parapsilosis													40	40	40*	40	110	110*
Candida krusei	5	5	10*	5	10	10*	10	20	20*	20	20	20 (40)	40	40	80*	40	80	80
Candida glabrata													20	80	80*	100	110	240
Cryptococcus neoformans	5	5	5	5	5	5	10	20	20	20	40	40	40	40	40	40	80	100
Cryptococcus gatti													10	20	80	2.5	110	160

* Indicates taht chitosan MIC and MFC were coincided

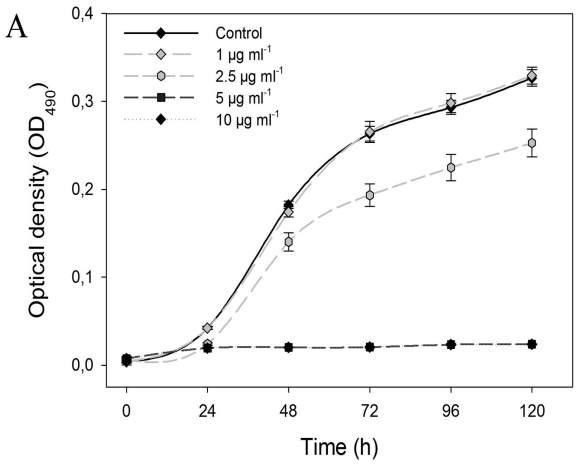
( ) Indicates that chitosan inhibitory concentration was fungicidal (MFC)

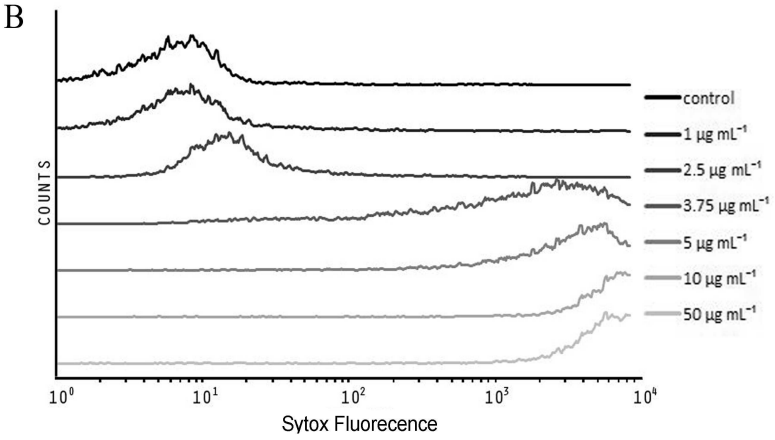
¥ Nutrients (C and N) was included in PDB medium at 0.04C-0.0015N g L^{-1}, 0.2C-0.007N g L^{-1}, 0.4C-0.015N g L^{-1}, 0.8C-0.031N g L^{-1}, 2.32C-0.087N g L^{-1}, 4C-0.15N g L^{-1}.

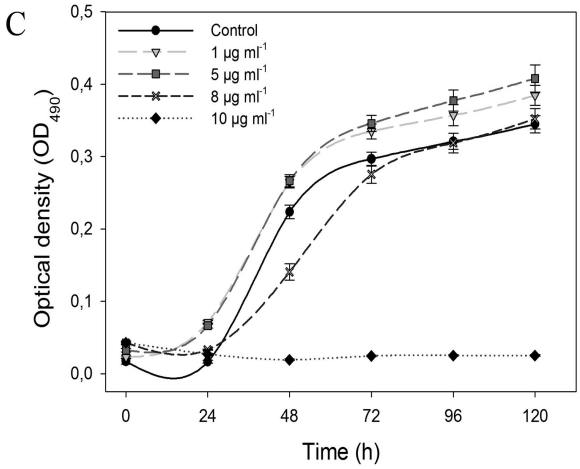
§ Nutrients (C and N) was included in YPD medium at 0.04C-0.015N g L^{-1}, 0.2C-0.074N g L^{-1}, 0.4C-0.15N g L^{-1}, 0.8C-0.3N g L^{-1}, 2C-0.74N g L^{-1}, 4C-1.5N g L^{-1}.

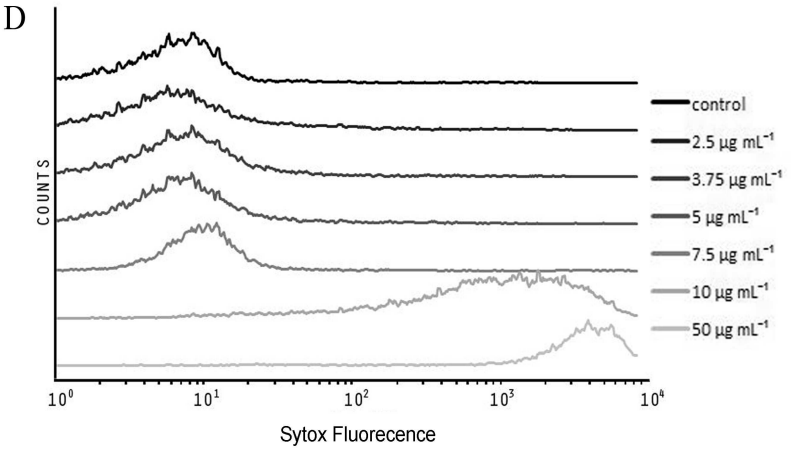
"ni" non-inhibitory

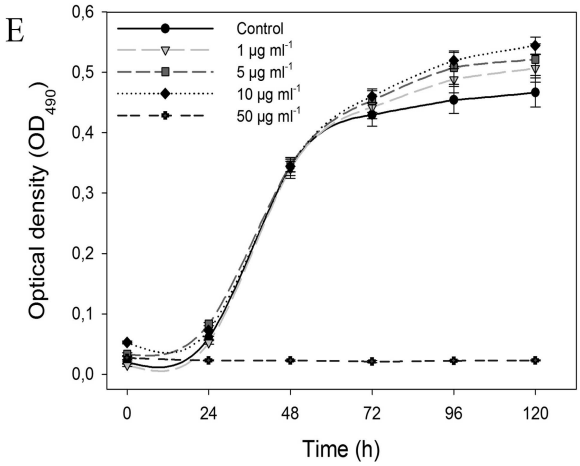
"-" not tested

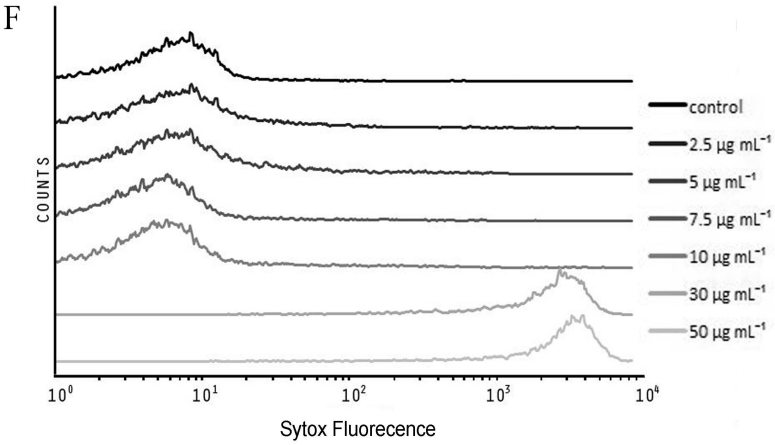

A
Control
1 µg ml-1
2.5 µg ml-1
5 µg ml-1
10 µg ml-1
Optical density (OD490)
Time (h)


B
control
1 µg mL-1
2.5 µg mL-1
3.75 µg mL-1
5 µg mL-1
10 µg mL-1
50 µg mL-1
COUNTS
Sytox Fluorecence

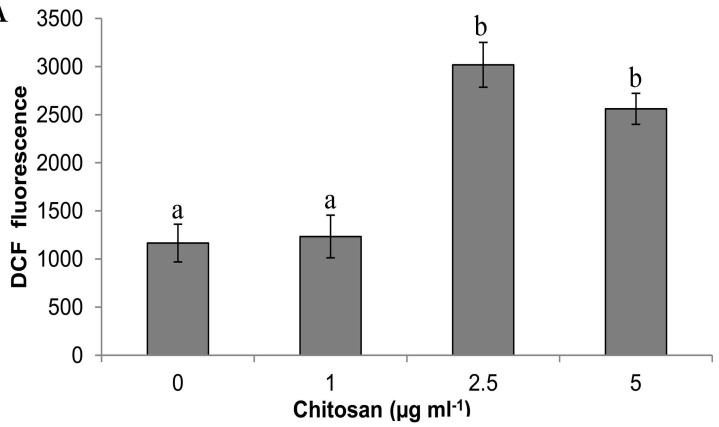

C
Control
1 µg ml-1
5 µg ml-1
8 µg ml-1
10 µg ml-1
Optical density (OD490)
Time (h)


D
control
2.5 µg mL-1
3.75 µg mL-1
5 µg mL-1
7.5 µg mL-1
10 µg mL-1
50 µg mL-1
COUNTS
Sytox Fluorecence

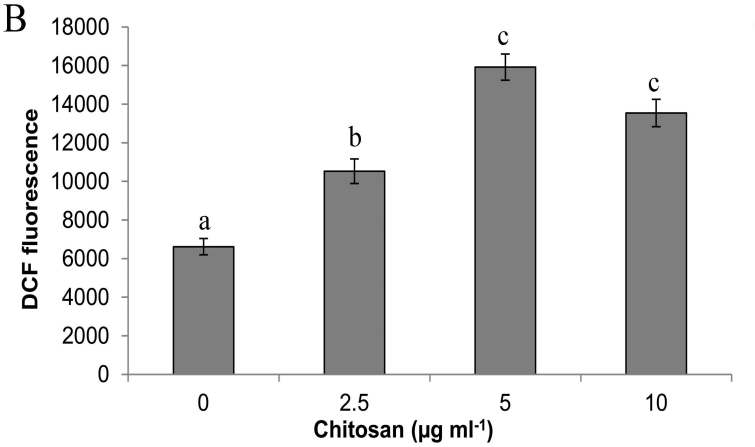

E
Control
1 µg ml-1
5 µg ml-1
10 µg ml-1
50 µg ml-1
Optical density (OD490)
Time (h)


F
control
2.5 µg mL-1
5 µg mL-1
7.5 µg mL-1
10 µg mL-1
30 µg mL-1
50 µg mL-1
COUNTS
Sytox Fluorecence

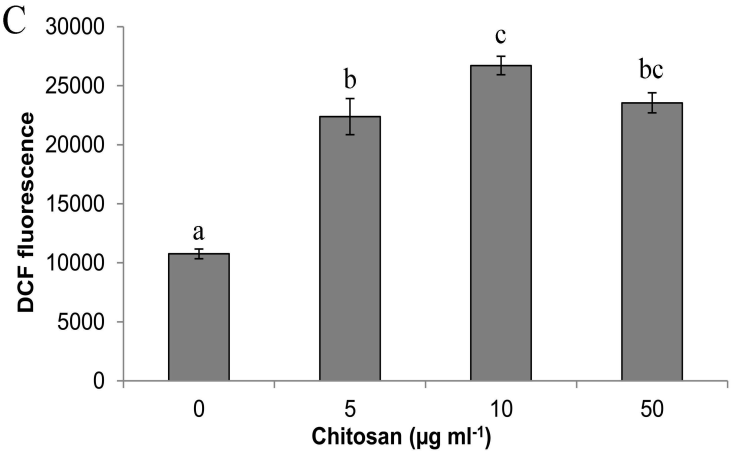
A

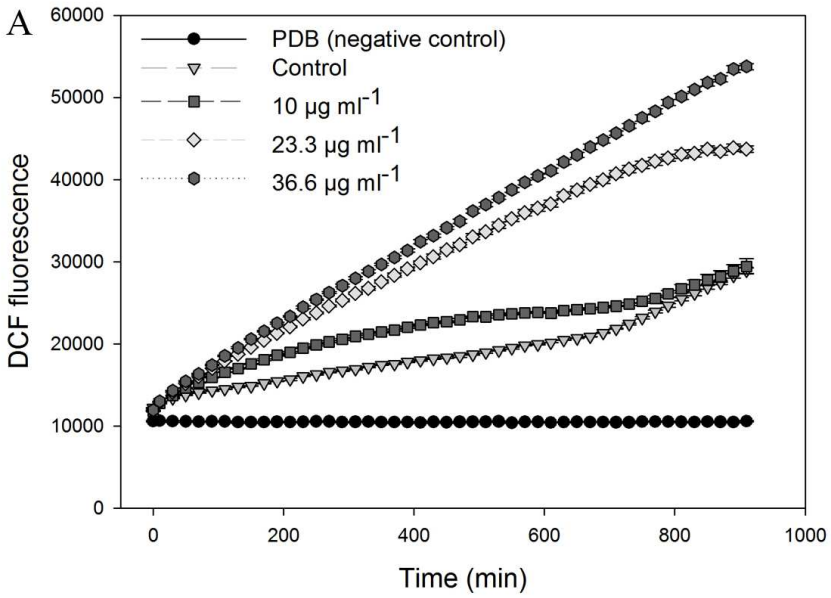

DCF fluorescence
3500
3000
2500
2000
1500
1000
500
0
a
a
b
b
0
1
2.5
5
Chitosan (µg ml-1)

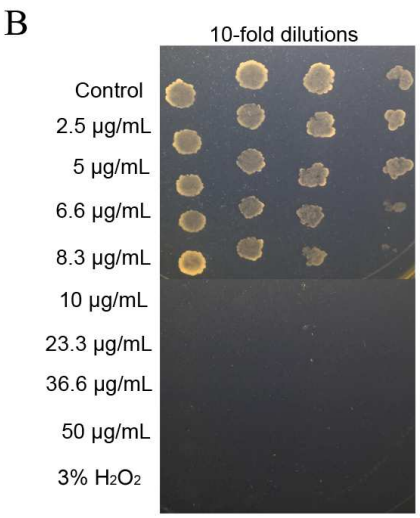
B

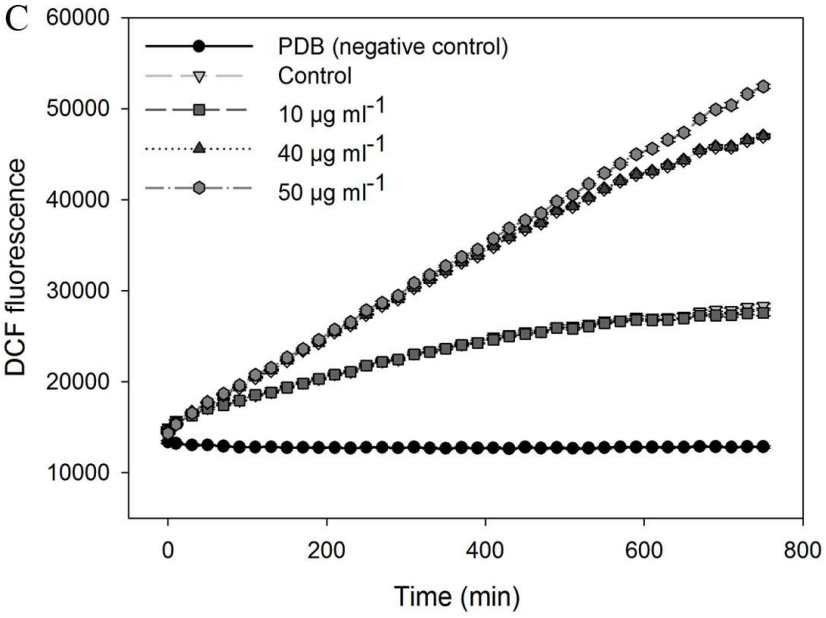

DCF fluorescence
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
a
b
c
c
0
2.5
5
10
Chitosan (µg ml-1)

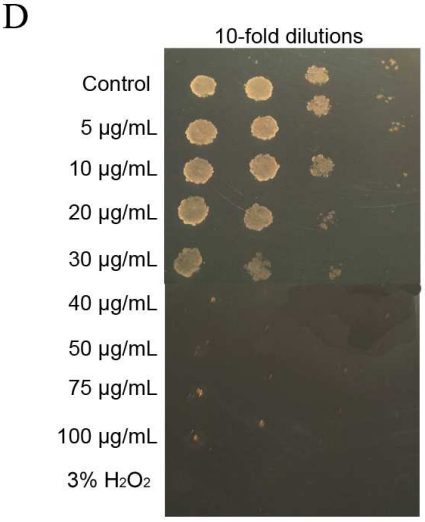
C

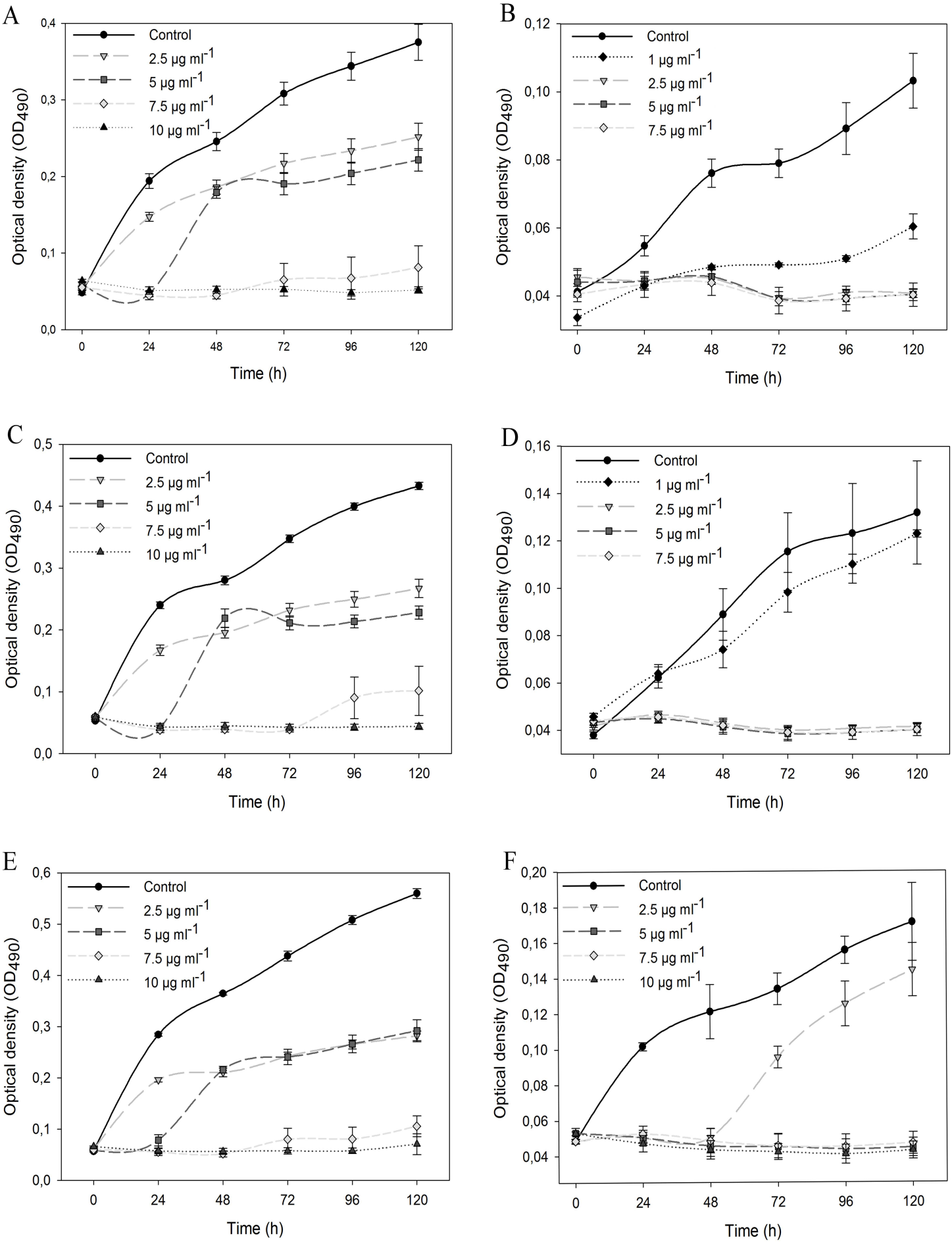

DCF fluorescence
30000
25000
20000
15000
10000
5000
0
a
b
c
bc
0
5
10
50
Chitosan (µg ml-1)

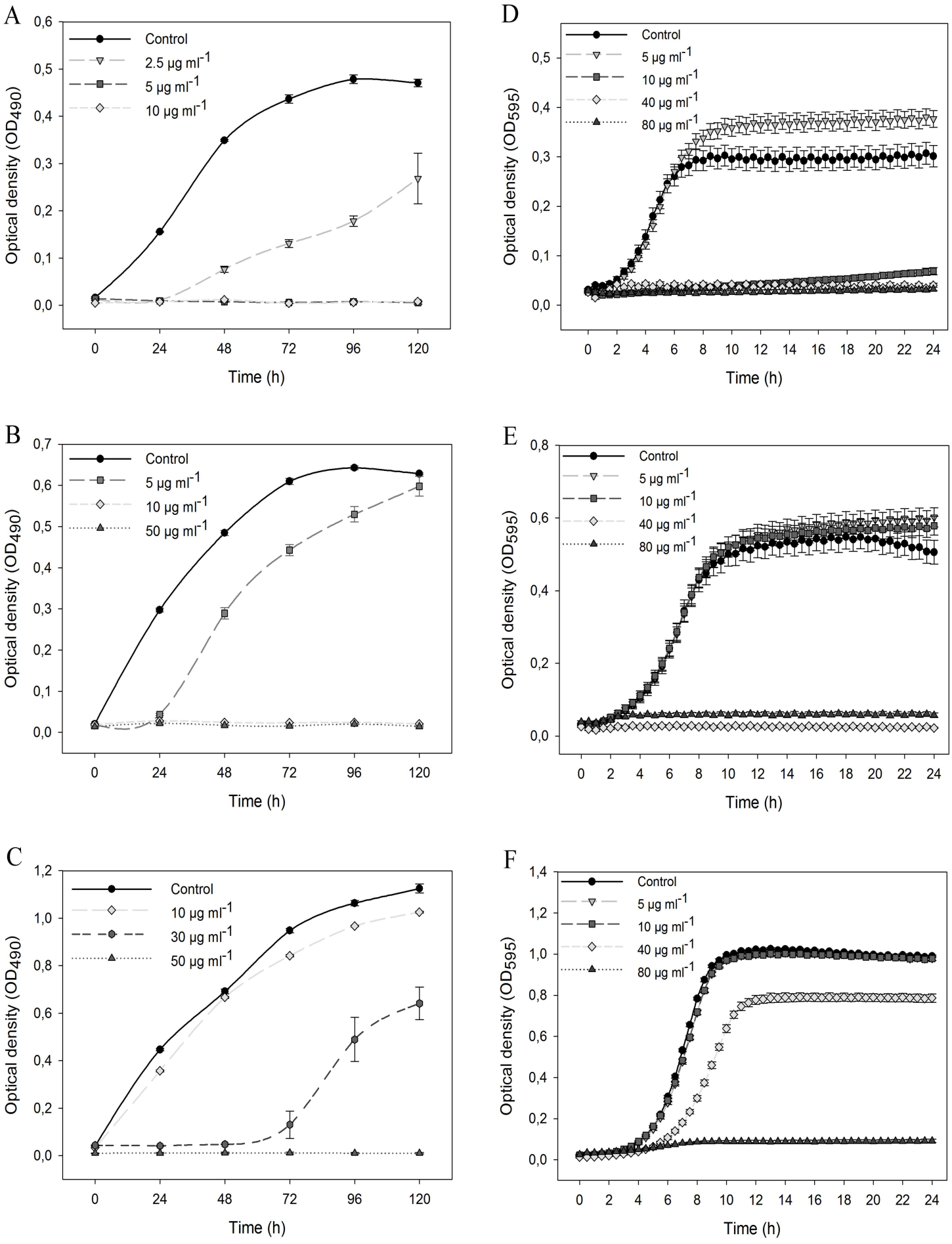

A
PDB (negative control)
Control
10 µg ml-1
23.3 µg ml-1
36.6 µg ml-1
DCF fluorescence
Time (min)
0
10000
20000
30000
40000
50000
60000
0
200
400
600
800
1000

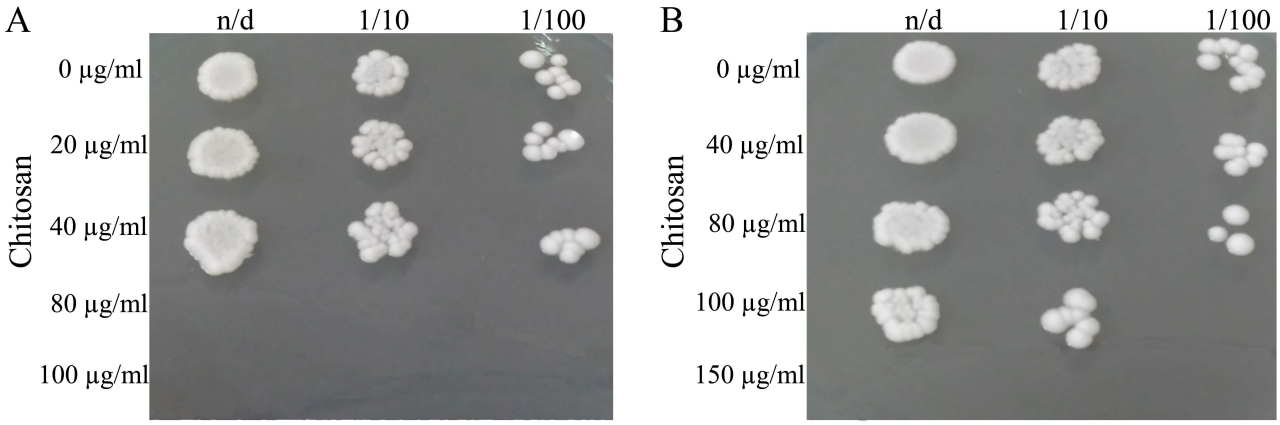

B
10-fold dilutions
Control
2.5 µg/mL
5 µg/mL
6.6 µg/mL
8.3 µg/mL
10 µg/mL
23.3 µg/mL
36.6 µg/mL
50 µg/mL
3% H2O2

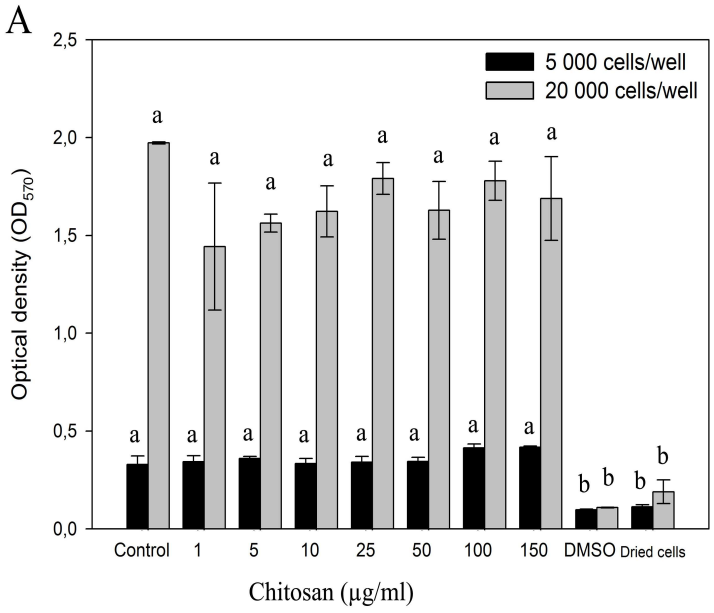

C
PDB (negative control)
Control
10 µg ml-1
40 µg ml-1
50 µg ml-1
DCF fluorescence
Time (min)
10000
20000
30000
40000
50000
60000
0
200
400
600
800

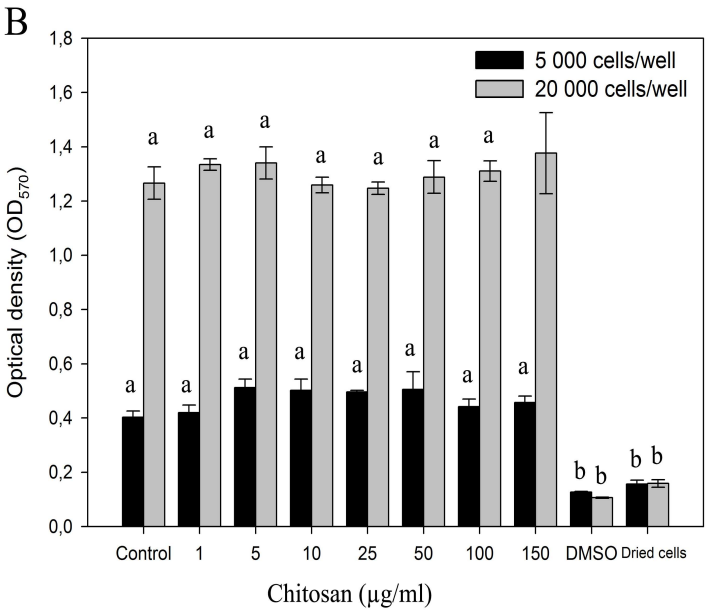

D
10-fold dilutions
Control
5 µg/mL
10 µg/mL
20 µg/mL
30 µg/mL
40 µg/mL
50 µg/mL
75 µg/mL
100 µg/mL
3% H2O2

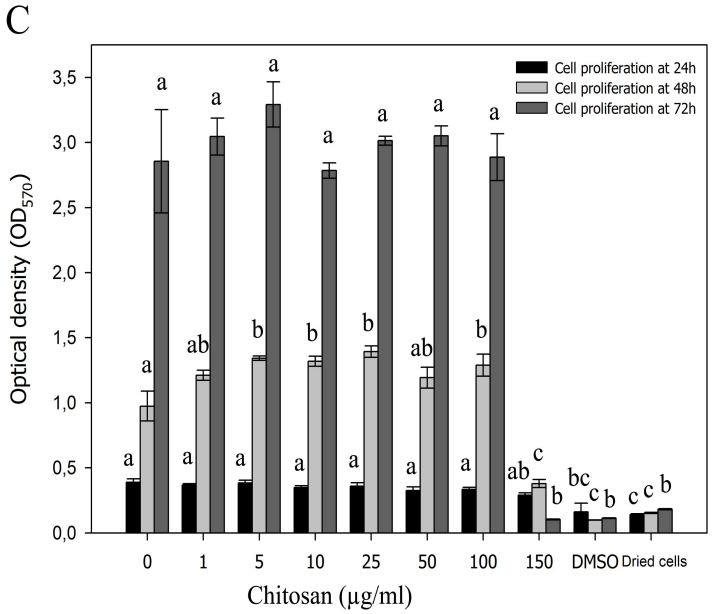

A
Control
2.5 µg ml-1
5 µg ml-1
7.5 µg ml-1
10 µg ml-1
Optical density (OD490)
Time (h)
B
Control
1 µg ml-1
2.5 µg ml-1
5 µg ml-1
7.5 µg ml-1
Optical density (OD490)
Time (h)
C
Control
2.5 µg ml-1
5 µg ml-1
7.5 µg ml-1
10 µg ml-1
Optical density (OD490)
Time (h)
D
Control
1 µg ml-1
2.5 µg ml-1
5 µg ml-1
7.5 µg ml-1
Optical density (OD490)
Time (h)
E
Control
2.5 µg ml-1
5 µg ml-1
7.5 µg ml-1
10 µg ml-1
Optical density (OD490)
Time (h)
F
Control
2.5 µg ml-1
5 µg ml-1
7.5 µg ml-1
10 µg ml-1
Optical density (OD490)
Time (h)

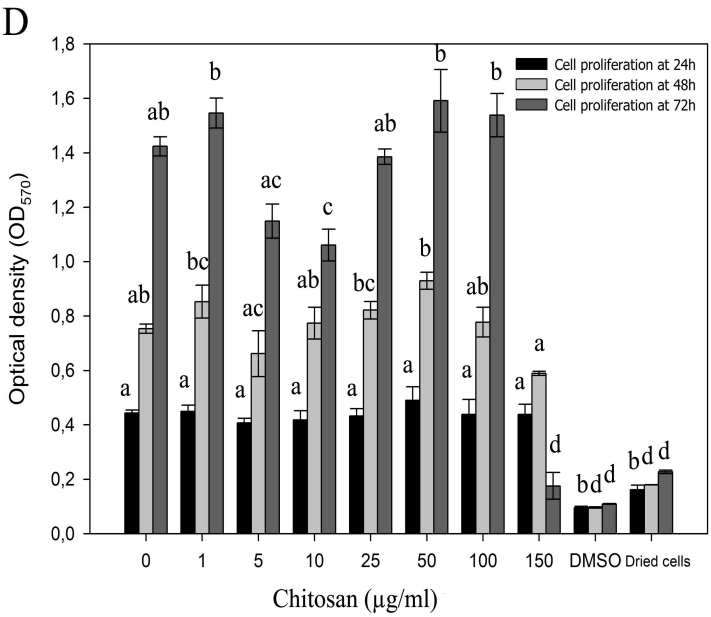

Fusarium proliferatum
Candida albicans
A
B
C
D
E
F
Control
2.5 µg ml-1
5 µg ml-1
10 µg ml-1
30 µg ml-1
40 µg ml-1
50 µg ml-1
80 µg ml-1
Optical density (OD490)
Optical density (OD595)
Time (h)

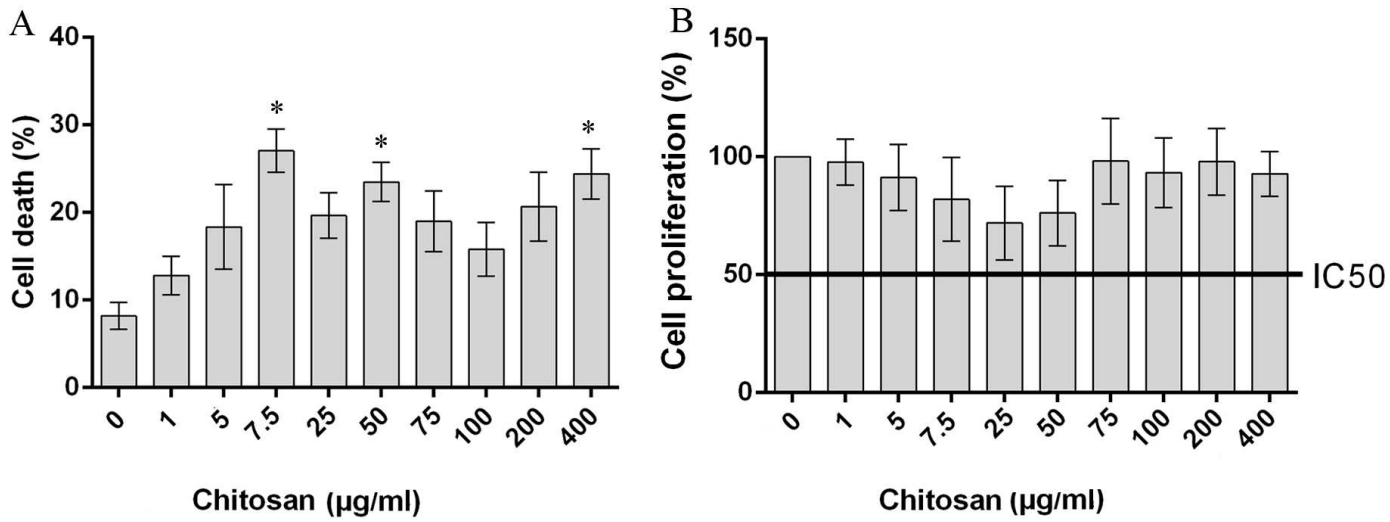

A
n/d
1/10
1/100
Chitosan
0 μg/ml
20 μg/ml
40 μg/ml
80 μg/ml
100 μg/ml
B
n/d
1/10
1/100
Chitosan
0 μg/ml
40 μg/ml
80 μg/ml
100 μg/ml
150 μg/ml


A
5 000 cells/well
20 000 cells/well
Optical density (OD570)
Control 1 5 10 25 50 100 150 DMSO Dried cells
Chitosan (µg/ml)

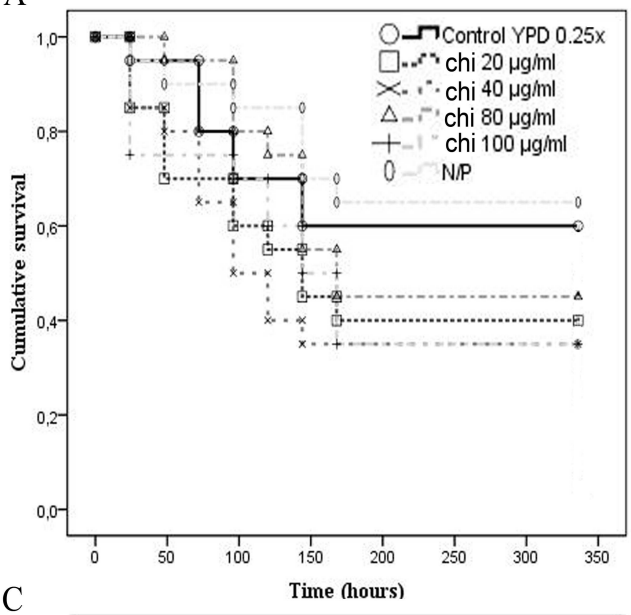

B
5 000 cells/well
20 000 cells/well
Optical density (OD570)
Control 1 5 10 25 50 100 150 DMSO Dried cells
Chitosan (µg/ml)


C
Cell proliferation at 24h
Cell proliferation at 48h
Cell proliferation at 72h
Optical density (OD570)
0 1 5 10 25 50 100 150 DMSO Dried cells
Chitosan (µg/ml)

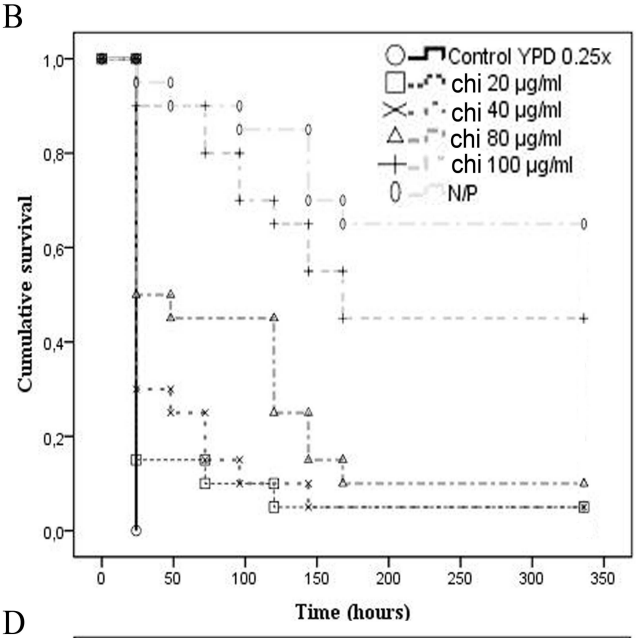

D
Cell proliferation at 24h
Cell proliferation at 48h
Cell proliferation at 72h
Optical density (OD570)
0 1 5 10 25 50 100 150 DMSO Dried cells
Chitosan (µg/ml)


A
Cell death (%)
40
30
20
10
0
*
*
*
0
1
5
7.5
25
50
75
100
200
400
Chitosan (µg/ml)
B
Cell proliferation (%)
150
100
50
0
IC50
0
1
5
7.5
25
50
75
100
200
400
Chitosan (µg/ml)

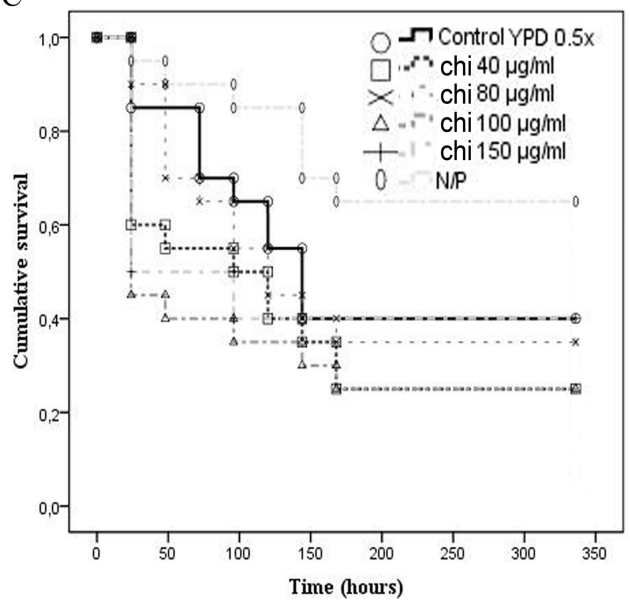
A


Control YPD 0.25x
chi 20 µg/ml
chi 40 µg/ml
chi 80 µg/ml
chi 100 µg/ml
N/P
Cumulative survival
Time (hours)

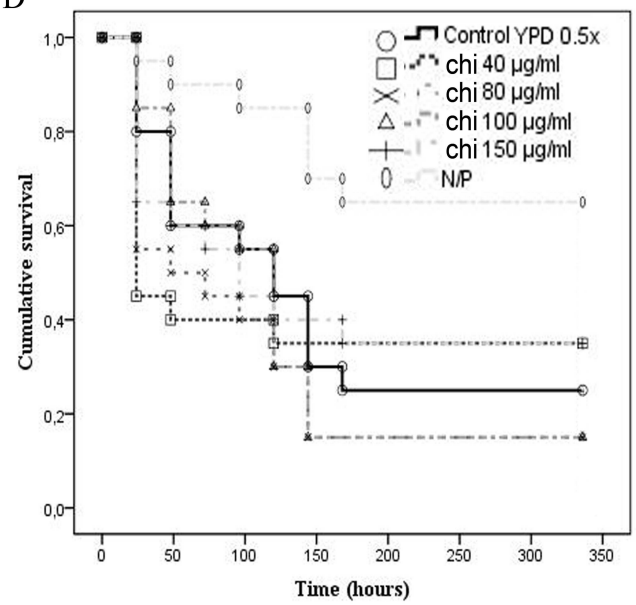
B


Control YPD 0.25x
chi 20 µg/ml
chi 40 µg/ml
chi 80 µg/ml
chi 100 µg/ml
N/P
Cumulative survival
Time (hours)

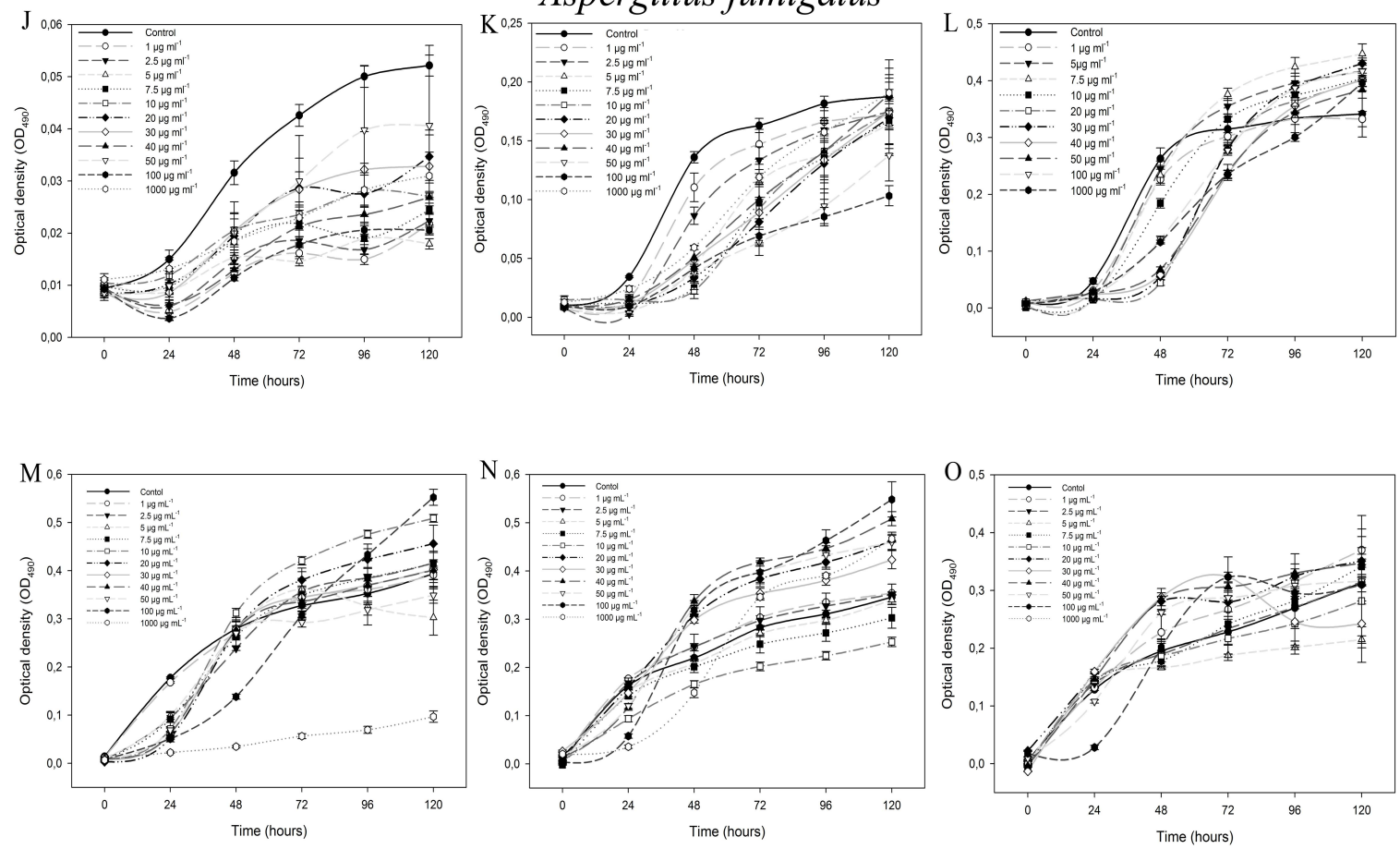
C

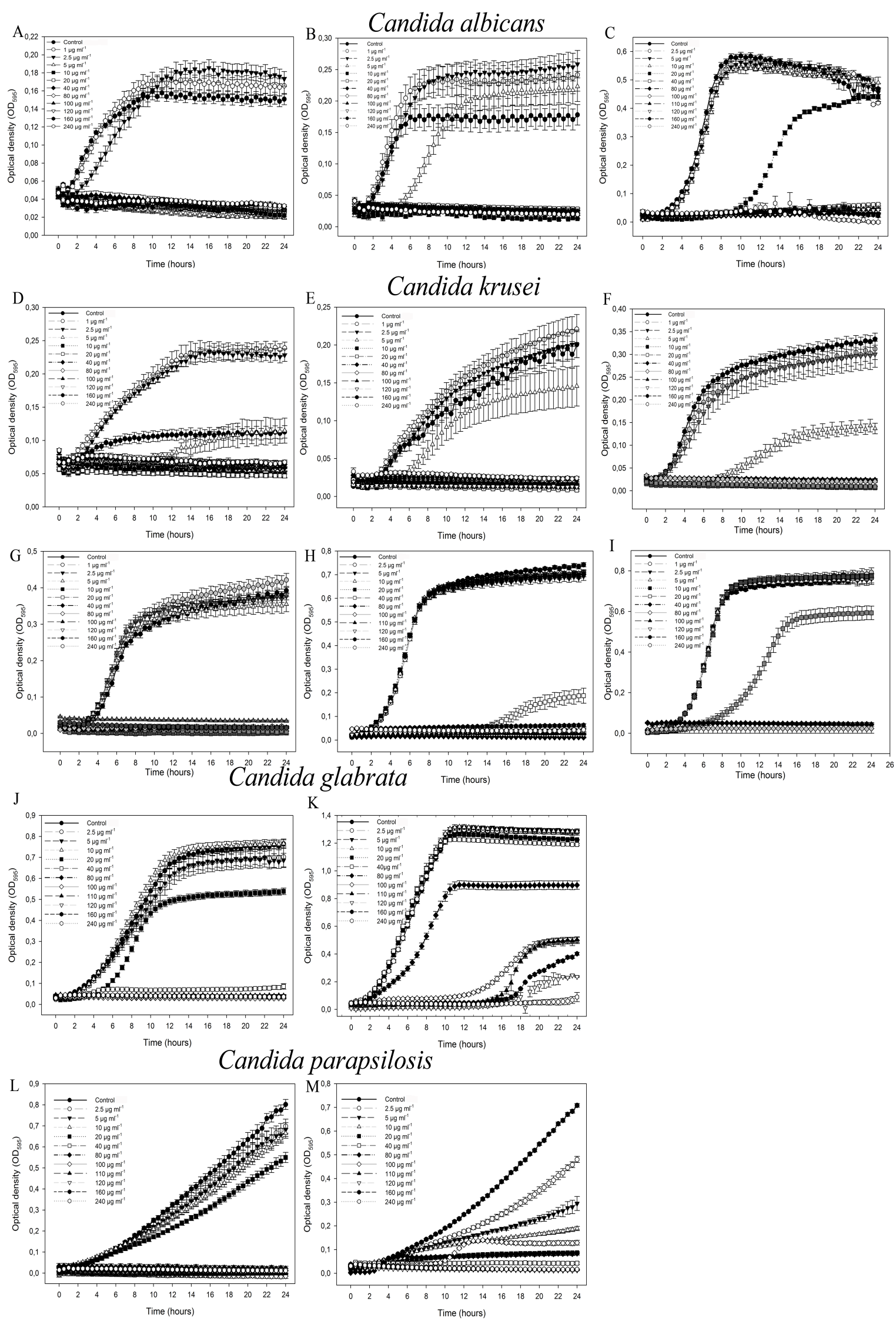

Control YPD 0.5x
chi 40 µg/ml
chi 80 µg/ml
chi 100 µg/ml
chi 150 µg/ml
N/P
Cumulative survival
Time (hours)

D

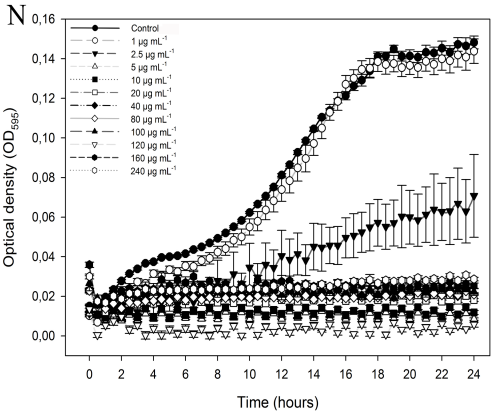

Control YPD 0.5x
chi 40 µg/ml
chi 80 µg/ml
chi 100 µg/ml
chi 150 µg/ml
N/P
Cumulative survival
Time (hours)

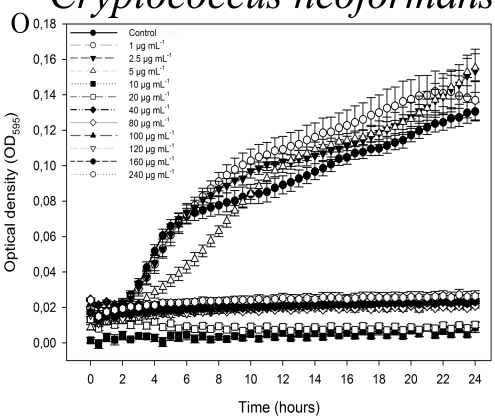
Aspergillus fumigatus

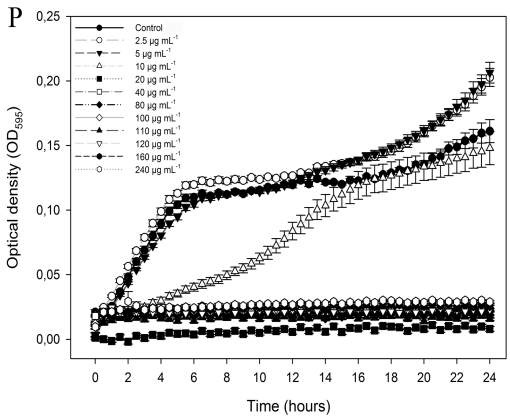


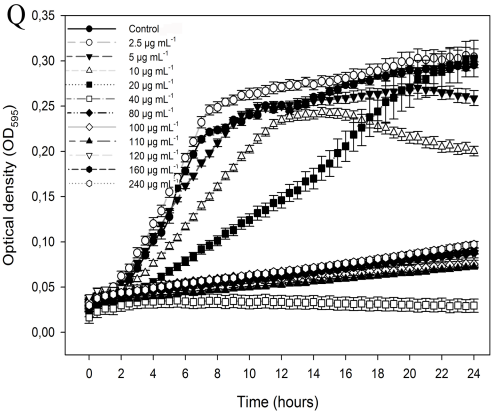

Candida albicans
Candida krusei
Candida glabrata
Candida parapsilosis
A
B
C
D
E
F
G
H
I
J
K
L
M
Optical density (OD595)
Time (hours)
Control
1 µg ml-1
2.5 µg ml-1
5 µg ml-1
10 µg ml-1
20 µg ml-1
40 µg ml-1
80 µg ml-1
100 µg ml-1
110 µg ml-1
120 µg ml-1
160 µg ml-1
240 µg ml-1

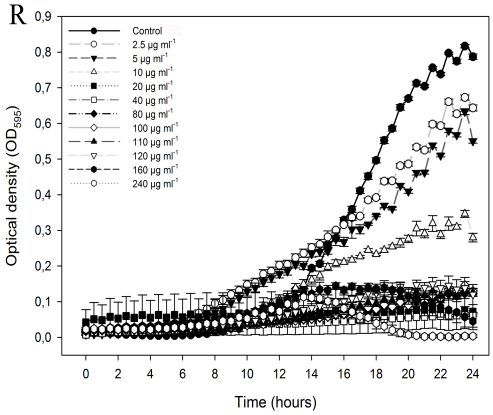
Cryptococcus neoformans

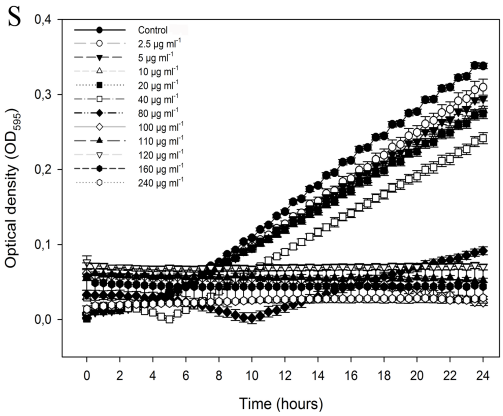




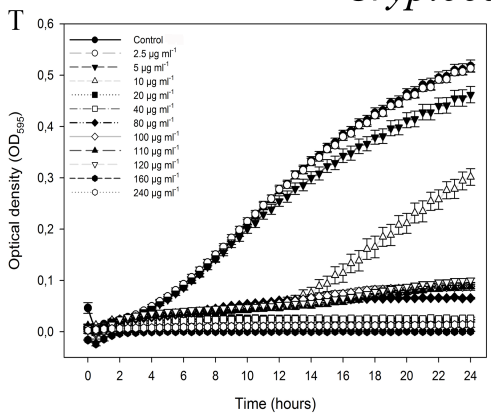


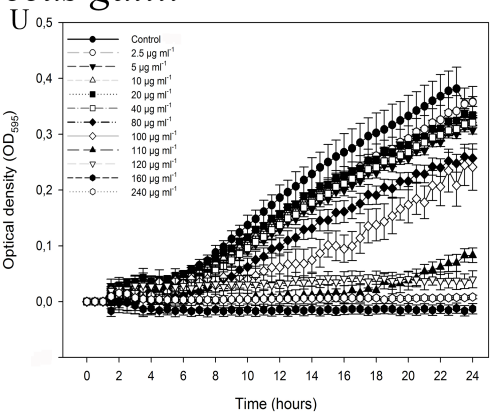


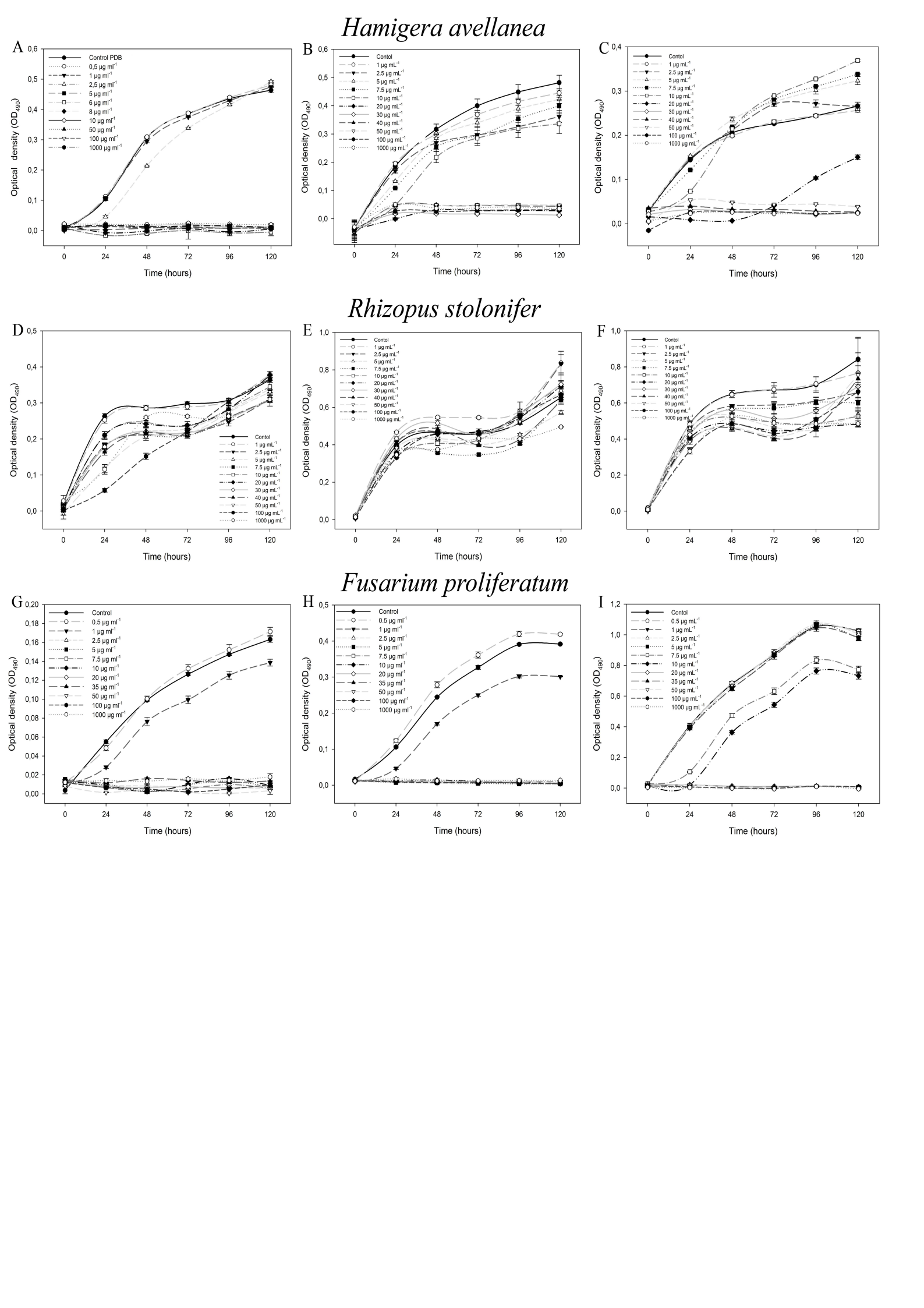




Cryptococcus gattii






Hamigera avellanea
Rhizopus stolonifer
Fusarium proliferatum
A
B
C
D
E
F
G
H
I
Optical density (OD490)
Time (hours)
Control PDB
Contol
Control
0,5 µg ml-1
1 µg ml-1
2,5 µg ml-1
5 µg ml-1
6 µg ml-1
8 µg ml-1
10 µg ml-1
50 µg ml-1
100 µg ml-1
1000 µg ml-1
0.5 µg ml-1
2.5 µg ml-1
7.5 µg ml-1
35 µg ml-1
1 µg mL-1
2.5 µg mL-1
5 µg mL-1
7.5 µg mL-1
10 µg mL-1
20 µg mL-1
30 µg mL-1
40 µg mL-1
50 µg mL-1
100 µg mL-1
1000 µg mL-1

		HEK293		COS7	
		Cells per well		Cells per well	
		5 000	20 000	5 000	20 000
Chitosan (µg/ml)	1	-4.15	26.86	-4.05	-5.40
	5	-9.41	20.78	-26.86	-5.87
	10	-1.42	17.76	-24.38	0.53
	25	-3.54	9.22	-23.06	1.45
	50	-4.66	17.49	-25.45	-1.76
	100	-25.71	9.82	-9.59	-3.50
	150	-26.82	14.39	-13.47	-8.74
	DMSO	70.34	94.49	68.51	91.60
	Dried cells	65.69	90.39	61.16	87.47

The horizontal dashed line separates cytotoxic index values $>IC_{50}$

		HEK293			COS7		
		Time (h)			Time (h)		
		24h	48h	72h	24h	48h	72h
Chitosan (µg/ml)	1	5.32	-24.32	-6.65	-1.43	-13.23	-8.57
	5	1.03	-37.84	-15.29	8.20	12.17	19.31
	10	10.38	-35.37	2.47	5.79	-2.65	25.49
	25	7.63	-42.97	-5.56	2.41	-9.07	2.72
	50	16.21	-22.44	-6.84	-10.60	-23.36	-11.75
	100	13.72	-32.36	-1.12	1.05	-3.19	-8.05
	150	24.96	61.07	96.36	1.05	21.77	87.66
	DMSO	58.40	89.77	96.02	78.35	87.21	92.30
	Dried cells	63.72	84.19	93.59	63.61	76.15	84.04

The horizontal dashed line separates cytotoxic index values $>IC_{50}$